A COMPARISON OF CULTURED HUMAN DERMAL FIBROBLASTS DERIVED FROM TERMINAL AND VELLUS HAIR BEARING SKIN

Differences in the expression of inhibitors of apoptosis proteins, oestrogen receptors, and responses to oestradiol under normal and wound induced conditions

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Abstract

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Wounds heal better in skin with terminal hair follicles (large and pigmented) as opposed to those with vellus hair follicles (small and unpigmented), while dermal fibroblasts from different anatomical regions also exhibit phenotypical differences. Tissue repair requires a tight control of cell proliferation, migration and apoptosis, and recent studies have shown the importance of inhibitors of apoptosis proteins (IAPs), which are proteins that prevent the process of apoptosis via their interaction with caspase molecules in wound healing. Oestrogens improve the rate and quality of wound healing, but their relationship with IAPs in human skin has not been studied. Therefore, terminal (scalp) and vellus (facial) hair bearing skin from the same donor was compared *in situ* and matching primary cultures of dermal fibroblasts were established from terminal (DF(T)) and vellus (DF(V)) hair bearing skin.

Using immunofluorescent staining, the expression of IAPs and their antagonists was compared at different stages of the hair cycle following depilation using a murine model and then in terminal and vellus hair bearing human skin. The size and granularity of matching DF(T) and DF(V) cultures was compared by FACS analysis and mRNA and protein expression of Apollon, cIAP2, NAIP and XIAP and their antagonists DIABLO and Xaf1 analysed by qRT-PCR and immunocytochemistry in unwounded and mechanically wounded fibroblast cultures. Differences in proliferation, migration, viability and caspase 3 activity in the presence of 17β-oestradiol and changes in mRNA expression of the oestrogen receptors (GPR30, ERα and ERβ) were compared between the two cell types.

IAP protein expression was generally found higher during mid anagen of the hair cycle in murine skin and hair follicles. Overall, expression was slightly higher in human terminal hair bearing skin compared to corresponding vellus hair bearing skin. IAP protein expression was similar in unwounded DF(T) and DF(V) cells with the exception of Apollon which was higher in DF(V) cells. With the exception of XIAP and its direct antagonist Xaf1, mRNA expression was higher in DF(V) cells compared to corresponding DF(T) cells. FACS analysis demonstrated that DF(V) cells were more granular than matching DF(T) cells and proliferated faster. 17β-oestradiol accelerated migration of DF(T) cells only. Mechanical wounding decreased XIAP mRNA in DF(T) and increased it in DF(V) cells, while simultaneously decreasing Xaf1 expression. In unwounded cells, 17β-oestradiol stimulated the expression of XIAP mRNA in both DF(T) and DF(V) cells, but in scratched monolayers, while it also increased expression in DF(T) cells it decreased it in DF(V) cells. A XIAP inhibitor reduced cell viability in both DF(T) and DF(V) cells, which was rescued by 17β-oestradiol in unwounded and mechanically wounded DF(T) cells, but only in unwounded DF(V) cells. 17β-oestradiol decreased caspase 3 activity in the presence of a XIAP inhibitor only in DF(T) cells.

These results demonstrate significant differences between dermal fibroblasts cultured from terminal and vellus hair bearing skin of the same individual. The correlation between an increase in XIAP in response to 17β-oestradiol and a higher number of viable cells, along with a reduction in caspase 3 activity suggests that the protective effect of 17β-oestradiol may be modulated via the regulation of XIAP. Further elucidation of these different signalling pathways in dermal fibroblasts from hair bearing skin may lead to improved therapies for chronic non-healing wounds, particularly in postmenopausal females.

Key Words: Wound Healing, Inhibitor of Apoptosis Proteins (IAPs), Oestrogen, Hair Follicle

Dedication:

To my Mother Jaona, Father Mumtaz, Brother Omar, Sister-in law Roaa, Grandmother Sabria and Great Uncle Yaseen

To my Best Friends Kara and Hazel and to their Parents

To Prof. Bob Bucher and his amazing wife Rosemary Bucher

To all whom are close in my heart.

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Abbreviations:

AF	Activating Factor
AIF	Apoptosis Inducing Factor
Ang	Angiopoietin
AP	Activator Protein
Apaf-1	Apoptotic Protease Activating Factor-1
Arts	Apoptosis related protein in the TGFβ signalling pathway
ATP	Adenosine TriPhosphate
BIR	Baculoviral IAP Repeat
BIRC	Baculoviral IAP repeat-containing protein
BMP	Bone Morphogenetic Proteins
cAMP	Cyclic Adenosine MonoPhosphate
CARD	Caspase Recruitment Domain
cIAP	Cellular IAP
c-RAF	Cellular Rapidly Accelerated Fibrosarcoma
DHEA	Dehydroepiandrosterone
DIABLO	Direct IAp Binding with LO pl
DISC	Death-Inducting Signalling Complex
DMEM	Dulbecco's Modified Eagle Medium
Dp	Depilated day
DP	Dermal Papilla
E_1	Oestrone
E_2	Oestradiol
E_3	Oestriol
E_4	Oestetrol
ECM	Extra Cellular Matrix
Eda	Ectodysplasin
Edar	Ectodysplasin Receptor
EGF	Epidermal Growth Factor
ER	Oestrogen Receptor
ERE	Oestrogen Response Elements
FADD	Fas-Associated protein with Death Domain
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
GF	Growth Factor
GPR30/GPER1	G-Protein coupled Receptor 30/ G-Protein Oestrogen Receptor 1
HtrA2	High temperature recruitment protein A2
IAP	Inhibitor of Apoptosis Protein
IBM	IAP Binding Motif

IGF	Insulin-like Growth Factor
ILP	IAP Like Protein
IRS	Inner Root Sheath
Iκβ	Inhibitor of κβ
JNK	Jun amino terminal kinase
LRR	Leucine Rich Repeat
MAPK	Mitogen-Activated Protein Kinase
MIF	Migration Inhibitory Factor
ML-IAP	Melanoma IAP
MUSE	Multi-lineage differentiating Stress Enduring
NAIP	Neuronal Apoptosis Inhibitory Protein
NFκβ	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NO	Nitric Oxide
NOD	Nucleotide binding Oligomerisation Domain
ORS	Outer Root Sheath
PDGF	Platelet Derived Growth Factor
Pen/Strep	Penicillin/Streptomycin
RING	Really Interesting New Gene
RIP	Receptor Interacting Protein
SHG	Secondary Hair Germ
SMA	Spinal Muscular Atrophy
Smac	Second Mitochondria-derived Activator of Caspase
TAK1	Transforming growth factor-β-Activated Kinase
TGF	Transforming Growth Factor
TGFβ	Transforming Growth Factor β
TIMP	Tissue Inhibitor of MetaloProteases
TNF	Tumour Necrosis Factor
TNF-R	Tumour Necrosis Factor Receptor
TRADD	TNF Receptor Associated Death Domain
TRAF	TNF Receptor Associated Factor
TRAIL	TNF Related Apoptosis Inducing Ligand
VEGF	Vascular Endothelial Growth Factor
Xaf1	XIAP Associated Factor 1
XIAP	X-chromosome linked IAP

Reagents and Suppliers:

Reagent	Supplier	Code
17β-oestradiol	Sigma	E2758
Antibodies:		
Apollon	Abcam	Ab19609
cIAP1	Abcam	Ab108361
cIAP2	Abcam	Ab32059
NAIP	Santa Cruz	Sc-11062
XIAP	Abcam	Ab21278
DIABLO	Imgenex	Img5753
Xaf1	Imgenex	Img379
ERα Antibody (HC-20)	Santa Cruz	sc-543
ERβ Antibody (H-150)	Santa Cruz	sc-8974
Donkey polyclonal Secondary Antibody to Rabbit IgG - H&L (FITC)	Abcam	Ab98502
Caspase 3 inhibitor	Millipore	235423-1mg
Caspase-Glo 3/7 Assay	Promega	G8090
DMEM (- phenol red)	Sigma	D5921
DMEM (+ phenol red)	Sigma	D6046
DMSO	Sigma	D8418
Donkey Serum	Sigma	D9663
Embelin	Sigma	E1406-10MG
Fast SYBR® Green Master Mix	Life Technologies	4385612
Fetal Bovine Serum	GIBCO	10270106, 41F1213K
Fetal Bovine Serum, Charcoal Stripped, USDA Approved Regions	Invitrogen	12676-011
ImProm-II™ Reverse Transcription System	Promega	A3800
Mitomycin C from Streptomyces caespitosus powder, BioReagent, suitable for cell culture (2mg)	Sigma	M4287-2MG
PBS	Sigma	p5493
Poly-L-Lysine	Sigma	P8920-500mL
Primers	Sigma	-
RNeasy Mini Kit (50)	Qiagen	74104
SACPIC Staining:		
Celestine Blue	Sigma	206342-25G
Histoclear	National-Diagnostics	HS-202
Indigo Carmine	Sigma	I8130-25G
Picric Acid	Sigma	P6744
Safrinin O	Sigma	S2255-100G
Trypan Blue	Sigma	T6146
UltraPure™ DNase/RNase-Free Distilled Water – 500ml	GIBCO	10977035

Chapter 1

BACKGROUND TO THESIS

1 Background to Thesis

1.1 Human Skin

One of the more complex organs of the human body is the skin (the integumentary system) (Kanitakis, 2002) which accounts for around 15% of the total weight of an adult. This organ can be divided into two main sections; the cutaneous membrane and the skin appendages.

1.1.1 Function

The skin has many important roles; it is the first line of defence against environmental stress such as microorganisms, UV rays and physical damage. Other roles of skin include temperature regulation by means of excreting sweat from the body, water balance, a waterproof barrier provided by the thin outer layer made up of stratified squamous epithelial cells, biochemical synthesis including Vitamin D and melanin. The skin also contains Merkel cells which are receptor cells that relay pressure sensations from the skin surface. Compression of these cells causes the release of chemical signals that stimulate sensory nerve endings (Lucarz and Brand, 2007). Another important function of the skin is to provide quick immune responses against infection; the cells responsible for this are the Langerhans cells (Martini and Ober, 2006).

1.1.2 Structure

The structure of the skin can be categorised into three different layers; they are: the epidermis which is the upper layer of the skin, under that the dermis; which is separated from the epidermal layer by a dermal-epidermal junction; and the inner layer is the hypodermis (Prost-Squarcioni, 2006). The origins of the layers differ, as embryologically the epidermis originates from the ectoderm, whilst both the dermis and the hypodermis originate from the mesoderm (Kanitakis, 2002). The skin appendages include the hair follicle, sebaceous gland, apocrine glands, multicellular exocrine glands and the nail.

Both the cutaneous membrane and the skin appendages making up the integumentary system, interact together to provide the functions of the skin.

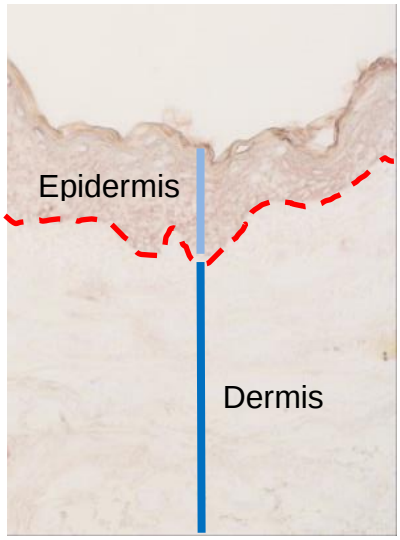


Figure 1.1: Epidermal and dermal layer of the human skin

Cytokeratin staining of a facelift skin sample from a 52 year old female patient. A clear distinction between the epidermis and dermis can be observed, dashed red line.

1.1.3 Epidermis

The epidermis is a multi-layered squamous epithelium consisting of five cellular layers where the thickness of those layers in humans varies between 0.05mm in the eyelids to around 1.5mm in the palms of the hand (Martini and Ober, 2006).

The different layers in order from the lower basal lamina are: stratum germinativum, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum (Martini and Ober, 2006); generally, there are only four layers found, however in thick skin, the stratum lucidum is also seen.

The epidermis is avascular; therefore for cells to survive, they depend on diffusion of nutrients from blood vessels found in the upper dermal layer of the skin. By default this separates the cells by their metabolic activity, cells that have the highest metabolism are found closest to the basal lamina, and those

with lower metabolism are found further away. The direction of differentiation of keratinocytes proceeds outwardly, where the cells migrate from the basal lamina (Fuchs, 1990a, b). There are four different cell types found in the epidermis, these include, keratinocytes, Merkel cells, melanocytes and Langerhans cells. The majority and the most abundant of these is the keratinocyte, which constitutes 90-95% of the cells of the epidermis. Different types of keratins are produced in the epidermis; keratin markers are used to distinguish between the keratin differentiated epidermal layers (Karantza, 2011; Sun et al., 1983). The remaining 5-10% of the epidermal cells are non-keratinocyte, this small percentage is made up of mainly melanocytes (>5%), Langerhans cells (>2%), and Merkel cells (>6%) (Kanitakis, 2002). Mast cells which are generally found in the dermis can also migrate into the basal layer of the epidermis (Harvima et al., 2008; James et al., 2011).

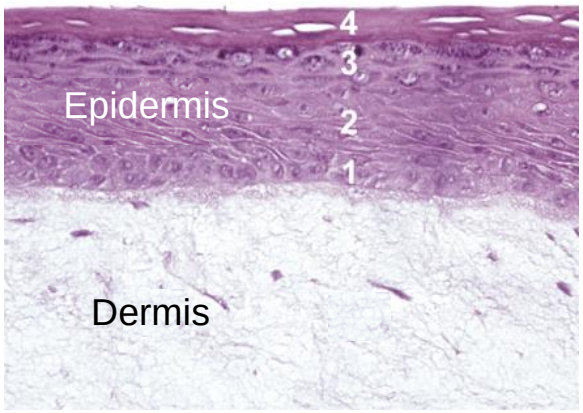


Figure 1.2: The epidermal layer of human skin

The epidermis is divided into four layers in human skin with an additional layer in thick skin such as palm skin; the layers from the innermost upwards are; 1- the stratum germinativum, 2- spinosum, 3- granulosum, and 4- corneum (Baba et al., 2005).

1.1.3.1 Stratum germinativum

The stratum germinativum is also known as the stratum basale. Hemi-desmosomes attach the cells in this layer to the basal lamina which separates the dermis from the epidermis. This layer is very important as it not only

separates, but also increases the strength of the bonds between the epidermis and the dermis (Martini and Ober, 2006).

Cells of the stratum germinativum are highly proliferative in order to maintain homeostasis as daughter cells from differentiating stem cells migrate upwards, transforming into transit amplifying cells (Barthel et al., 2000).

Merkel cells, which are specialised cells are also found in the stratum germinativum; the speciality of these cells are that they are able to detect pressure (Lucarz and Brand, 2007; Nakafusa et al., 2006). Melanocytes, which are pigment producing cells via the production of melanin are also found in this layer (Martini and Ober, 2006). The ratio of melanocytes to keratinocytes is 1:36 (Cichorek et al., 2013; De Luca et al., 1988; Fitzpatrick and Breathnach, 1963).

1.1.3.2 Stratum spinosum

Daughter cells from the cells found in the stratum germinativum are found in the stratum spinosum. There are 8-10 layers of keratinocytes bound together by desmosomes that make up the stratum spinosum. Cells that enter this layer from the stratum germinativum continue to divide, increasing the thickness of the epidermis. Keratin 10 is generally found in this layer as opposed to keratin 14 which is found in the stratum germinativum (Reichelt et al., 2004; Santos et al., 2002). The majority of the cells found in this layer are polyhedral keratinocytes, flattened as they migrate up in the epidermis (Arda et al., 2014). This layer also contains Langerhans cells, which play a role in the immune response system. Many studies have shown the immunostimulatory effect of Langerhans cells in the epidermis is via the stimulation of T cells (Romani et al., 2010; Romani et al., 2006). Another function of this layer is to retain lipids to prevent moisture loss via keratinocyte ultrastructure (Martini and Ober, 2006; Minematsu et al., 2011).

1.1.3.3 Stratum granulosum

In the stratum granulosum, there are approximately three-five layers of keratinocytes in human skin. In this layer of the epidermis, most of the cells have stopped dividing. Their main role is diverted to producing the proteins keratin and keratohyalin. Keratin is a tough, fibrous protein and is the basic structural component of skin and nails. With the development of these keratin proteins, the cells become thinner and flatter in appearance whilst their membranes become thicker and less permeable. The second protein keratohyalin forms dense cytoplasmic granules; these granules promote dehydration of the cell as well as cross linking with keratin (Martini and Ober, 2006). Lamellar granules, known also as keratinosomes are secretory granules essential for barrier formation (Odland and Holbrook, 1981).

1.1.3.4 Stratum lucidum

This layer is only found in the thick layers of human skin such as the fingertips, palms and soles. This layer consists of three to five layers of non-proliferating keratinocytes, which are clear and flattened; the keratinocytes are densely packed and filled with eleidin, which is an intermediate in thetransformation of keratohyalin to keratin (Saladin and Porth, 1998) and thick plasma membranes (Martini and Ober, 2006; Tortora and Derrickson, 2008). The difference between the stratum lucidum and the stratum corneum is that stratum lucidum has a pale eosinophilic appearance due to the lack of organelles as eleidin does not stain well with indistinct boundaries (Saladin and Porth, 1998); and a higher lipid content. The keratinocytes in both of these layers are anucleate (Busam, 2010).

1.1.3.5 Stratum corneum

This layer forms the upper layer of the epidermis, which is exposed to the environment. In humans the stratum corneum generally has 15-30 layers of keratinocytes, which are fully keratinized i.e. the cells are filled with keratin. Terminally differentiated cells at the surface of the epidermis are tightly

connected, they are shed as groups instead of individual cells (Martini and Ober, 2006).

The duration it takes for the cells to migrate from the inner stratum germinativum layer to the outer stratum corneum of the epidermis is generally 15-30 days depending on the location in the human body and the thickness of the skin (Candi et al., 2005). After the cells have reached the surface, they normally remain for a further 2 weeks before they are shed (Martini and Ober, 2006).

1.1.4 Dermis

This layer of the skin is found under the epidermis separated by a thin basement membrane zone (Martini and Ober, 2006). Its thickness ranges from 0.2mm found in the eyelids to 4mm on the back of adult humans. The dermis is composed of connective tissue; dermal fibroblasts found in this layer are responsible for the production of the extra cellular matrix (ECM), which consists predominantly of elastic and collagen fibres. The proteins elastin and fibrillin are produced by fibroblasts, and secreted to form the ECM (Haynes et al., 1997; Kajiya et al., 1997); these proteins are important for the formation of the elastic fibres and are essential in the production of the connective tissue. Microarray analysis of dermal fibroblasts derived from different anatomical regions and donors of different ethnicity has demonstrated that they can be differentiated from each other via the expression of specific genes (Bryant and Nix, 2012; Chang et al., 2002).

1.1.4.1 Dermal fibroblasts

The most important cell found in all dermal layers of the skin is the fibroblast; the fibroblast is responsible for producing the macromolecules that constitutes the ECM (Alberts, 2002). Fibroblasts are derived from the mesoderm, and on occasion, fibroblasts maybe derived from epithelial cells in an epithelial to mesenchymal transition, which is an important process in wound healing (Kalluri and Weinberg, 2009; Yan et al., 2010). Fibroblasts are responsible for the synthesis of collagens, glycosaminoglycans, reticular and elastic fibres,

and glycoproteins, which are all important components of the ECM. Dermal fibroblasts derived from different origins such as keloid, palmar, non-palmar and plantar do not show the same characteristics, even when cultured in the same culture conditions, such as TGFβ1-RII and αSMA expression, their size and granularity and their proliferation rate (Chipev and Simon, 2002).

Recent studies have identified a type of stem cell among dermal fibroblasts (Kuroda et al., 2010; Kuroda et al., 2013), which has been used to try to induce differentiation into specific cell types including melanocytes for therapeutic purposes (Tsuchiyama et al., 2013); specific isolation techniques have been established (Kuroda et al., 2013). Multi-lineage differentiating stress enduring (MUSE) cells have been identified in select dermal fibroblast populations (Wakao et al., 2011); these cells carry the properties of stress tolerance, expression of pluripotent markers, self-renewal and the ability to differentiate into the three different cell lineages, i.e. endodermal, mesodermal and ectodermal. As the name suggests, these cells appear under stress including long term trypsin incubation, and were isolated from cultures of normal dermal fibroblasts, which were stress induced, the remaining live cells were cultured in suspension to retain their stem cell properties. To ensure that these cells were the cells of interest, specific markers Nanog, Oct3/4, SSEA-3, PAR4 and Sox2 which are transcription factors involved in self renewal, were used for identification.

However, not all fibroblasts have the potential to be MUSE cells, the study conducted by Kuroda et al. (2010) showed that from the initial number of human skin fibroblasts cultured, only 11.6% of them were MUSE cells. These cells show reduced proliferation especially after 14 days when cultured in clusters (cell suspension), however when transferred to an adherent culture, they resumed their proliferative properties and were still able to form their distinct cluster after multiple “stress cycles” and retain the MUSE cell properties.

These MUSE clusters were transferred onto gelatine coated dishes and after 7 days, via immunofluorescent staining using specific markers, cells were

checked for lineage differentiation; Table 1.1 shows the percentage of cells that have differentiated into the different cell lineages using the specific markers. This demonstrates that MUSE cells have stem cell properties and could potentially be used to differentiate into other cell types.

Marker	Cell Lineage	Percentage cells
Neurofilamen-M	Ectodermal	3.5%
αSMA	Mesodermal	12.2%
Desmin	Mesodermal	14.2%
Afetoprotein	Endodermal	2.7%
Cytokeratin 7	Endodermal	5.5%

Table 1.1: Percentage of MUSE cells differentiating into different cell lineages

Five different cell markers for the three types of cell lineages, ectodermal, mesodermal and endodermal were used to analyse the percentage of MUSE enriched dermal fibroblasts differentiating into different cell lineage types (Kuroda et al., 2010).

The dermis can be split into two layers with no distinct boundary as seen between the epidermis and dermis; the papillary (upper) layer and the reticular (lower) layer (Martini and Ober, 2006). Their distinction is determined via the orientation, distribution and type of collagen found in the two layers; produced by fibroblasts found in the region. The components of the ECM are not uniform throughout the dermis, they vary between the papillary and reticular layers (Sorrell and Caplan, 2004). Different types of collagen are produced in the dermis; the most common are collagen type I and type III; a list of the collagens and where they are found in the dermis is given in Table 1.2. Production of collagen, especially type IV and type VII is influenced by keratinocytes via TGFβ2 signalling (Sorrell and Caplan, 2004). The amount of type I and type III collagen found in the dermis is reduced with age (Junqueira et al., 1983; Varani et al., 2006).

The dermis also contains, blood and lymphatic vessels, nerve fibres, glandular units including the sweat glands, hair follicles, sebaceous glands and nail

roots. The histology of the boundary between the epidermis and dermis is very distinct, with a wave like structure, where the upward extensions from the dermis are the dermal papillae and the downward extensions are from the epidermis, interlocking the two structures together, such that the layers do not slide free from each other; these are known as the rete ridges. Depending on the location on the human body and the thickness of the skin, the extensions from the dermis not only allow grip to be maintained, but will vary so as to allow access of blood nutrients to the outer surface of the skin (Saladin and Porth, 1998).

Matrix Component	Papillary Dermis	Reticular dermis
Collagens I and III	High ratio of type III to I	Low ratio of type III to I
Collagen IV	Present in basement membrane	Absent
Collagen VI	Present in dermal-epidermal junction (DEJ)	Weakly present
Collagen XII	Present	Low to absent
Collagen XIV	Low to absent	Present
Collagen XVI	Present in DEJ-region	Absent
Tenascin-C	Present in DEJ-region	Absent
Tenascin-X	Weak in DEJ-region	Present
Versican	Diffuse in DEJ-region, present in matrix fibrils	Present in association with elastic fibres
Decorin	Present	Present

Table 1.2: Distribution of collagen and other ECM compartments in the dermis

A comparison of the components of the ECM between the papillary dermis and reticular dermis adapted from (Sorrell and Caplan, 2004).

1.1.4.2 Papillary layer – upper dermis

The papillary layer is an area of loose connective tissue and forms a fifth of the total dermal thickness (Saladin and Porth, 1998). In human skin it is approximately 300-400µm in depth, depending on location and age (Sorrell and Caplan, 2004). This layer consists of the capillaries, the lymphatic and sensory nerve fibres that supply blood and fluids to the surface of the skin. Elastin is concentrated in this layer, with elastic sheets found near the reticular layer (Meyer et al., 2000; Starcher et al., 2005). Collagen fibres are found packed as thin bundles, with a diameter of 10µm per bundle (Ventre et al.,

2009). In this layer the fibre bundles are generally thin and randomly oriented (Sanders et al., 1999). The main type of collagen found in this layer of the dermis is type III collagen (Junqueira et al., 1983).

Human fibroblasts found in this region of the dermis are known to divide faster than fibroblasts cultured from the lower reticular layer of the dermis (Sorrell and Caplan, 2004). Also, an important role that the fibroblasts have is communicating with keratinocytes, particularly to form the basement membrane of the epidermis. However, site matched fibroblasts (papillary and reticular) interact differently with keratinocytes (Sorrell et al., 1996). Also, myofibroblasts from wound sites appear to interact less efficiently and show less support for keratinocyte differentiation and basement membrane formation, in comparison to fibroblasts from non-wounded sites (Moulin et al., 2000; Sorrell and Caplan, 2004).

Recently, markers have become available to distinguish the two dermal layers from each other, this has become important in studying the dermis under different conditions, such as during ageing (Janson et al., 2013). During ageing, there is a loss in the epidermal ridges accompanied by a loss in the number of fibroblasts in the papillary layer of the dermis. With the loss of fibroblasts, there are changes in the levels of some of the ECM components (Janson et al., 2013). Janson et al. (2013) hypothesised that papillary fibroblasts transformed into reticular fibroblasts, and compared the structure and characteristics of low and high passage fibroblasts derived from the papillary layer with matching patient low passage reticular fibroblasts. They observed that specific markers such as αSMA, reticular calponin 1 (CNN1), transglutaminase 2 (TGM2) and cadherin 2 (CDH2) and papillary podoplanin (PDPN), netrin 1 (NTN1) and C-C chemokine receptor type 11 (CCRL1) that are used to differentiate between the two dermal layers were altered, where papillary marker expression was reduced in high passage papillary fibroblasts and low passage reticular fibroblasts, whilst the expression of reticular markers was increased in high passage papillary fibroblasts.

1.1.4.3 Reticular layer – lower dermis

This layer of the dermis is constructed by a meshwork of dense irregular connective tissue where both collagen and elastic fibres are found and constitutes four fifths of the dermal thickness (Saladin and Porth, 1998). The collagen fibres found in the reticular layer are an entanglement of densely packed collagen bundles of more than 50µm in diameter forming the meshwork, and are mostly oriented parallel to the skin surface (Sanders et al., 1999; Ventre et al., 2009). The main type of collagen found in the reticular layer is type I collagen (Junqueira et al., 1983). In this layer, the anagen hair follicle bulb, the sebaceous glands and the functional units of the sweat glands are also located (Alberts et al., 2008).

Even though human fibroblasts cultured from the reticular layer of the dermis grow slower than those cultured from the papillary layer from the same site e.g. breast, they contract faster than fibroblasts from the upper papillary layer. In addition, they do not have the same properties of cell contact inhibition allowing cells to attain a higher density when cultured in a monolayer (Schafer et al., 1985; Sorrell et al., 1996) as reviewed by Sorrell and Caplan (2004).

1.1.5 Hypodermis

The hypodermis is a subcutaneous layer composed of fatty connective tissue and loose connective tissue and has a slightly elastic property. Generally the boundary that separates the dermis from the hypodermis is indistinct, with connective tissue from the reticular layer being extensively interwoven with the connective tissue in the hypodermis (Martini and Ober, 2006). The predominant cell types found in the hypodermis are adipocytes, although fibroblasts and macrophages are also seen. The role of the hypodermis is to provide insulation and also cushioning with added mobility (Bryant and Nix, 2012). Recently, a study by Wojciechowicz et al. (2013), demonstrated that the adipose layer was created from the cells found in the lower layer (reticular layer) of the dermis. This occurred at the stage of embryonic hair follicle

development (in mice), suggesting the importance of hair follicles in dermal development.

1.2 The Hair Follicle

The hair follicle is considered to be a mini-organ, which is very complex in both structure and function. Hair follicle development initiated by the formation of the placode occurs in early skin development; however, regeneration occurs throughout life (Chuong et al., 2007; Reynolds et al., 1999).

There are two main types of hair follicles found in the skin of the human body, however, a third type can be distinguished in the foetus (Narisawa et al., 1995); in the foetus, lanugo hair follicles are found which are very soft and unpigmented and are generally shed just prior to birth. They are replaced by terminal or vellus hair follicles depending on body site. In humans at puberty, with increased levels of sex hormones, vellus hair follicles may transform into terminal hair follicles in regions such as the axilla, and in males, on the face for example the beard; this takes place over several hair follicle cycles (Hamada and Randall, 2006). The locations of these hair follicles are quite distinct in the human body, and also vary between genders. Vellus hair follicles are small, fine and less pigmented, while terminal hair follicles produce large pigmented hairs found on the scalp, eye brows and eye lashes (Lai-Cheong and McGrath, 2013). Although the majority of the skin contains either terminal or vellus hair follicles, there are regions which contain no hair follicles, these include the lips, the back of the ear, the palms and soles of the hand and foot (Wosicka and Cal, 2010).

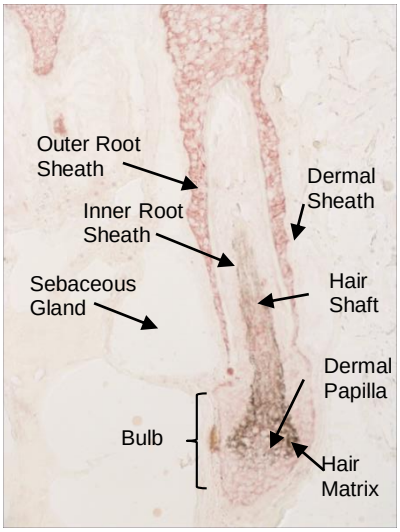


Figure 1.3: Structure of the Hair Follicle

Cytokeratin staining of a terminal hair follicle from female scalp (age, 52 years). The bulb region showing the hair matrix and dermal papilla surrounded by the dermal sheath. The shaft surrounded by the inner and outer root sheath.

1.2.1 Hair follicle function

The hair follicles have different roles in humans; these include additional sensory elements to the skin, temperature regulation and communication. Hair follicles are not only important in the production of hair; they are self-renewing and contain a reservoir of multipotent stem cells that help in the regeneration of the epidermis and are also important in wound healing (Millar, 2002).

1.2.2 Hair follicle structure

The entire structure of the hair follicle can be divided into two regions, the permanent region, which visibly does not appear to cycle, and the inferior region, which with each hair cycle is remodelled (Schneider et al., 2009; Stenn and Paus, 2001). There are many different components that make up the hair follicle, these are the hair shaft, inner root sheath, outer root sheath, the bulge (stem cell niche) found as part of the outer root sheath (Schneider et al., 2009), and located at the insertion of the arrector pili muscle, under the sebaceous

gland (Cotsarelis et al., 1990; Lyle et al., 1998), the companion layer found in between the inner outer root sheath, the bulb, which is formed by the mesenchymal dermal papilla surrounded by the epithelial hair matrix; the entire follicle is then surrounded by the connective tissue or dermal sheath. The growing hair follicle is a highly vascularised structure (Wosicka and Cal, 2010).

1.2.3 Hair follicle morphology

The development of the hair follicle is determined by a tightly coordinated interaction between the ectoderm and mesoderm (Fuchs, 2007; Schmidt-Ullrich and Paus, 2005). The initial formation of the hair follicle involves stem cells from different lineages, which are the ectoderm and mesoderm. Ectodermal stem cells give rise to epithelial components of the hair follicle including the sebaceous and apocrine glands. Whereas stem cells from the mesoderm give rise to the follicular dermal papilla and the connective tissue; melanocytes from the neural crest are responsible for the pigmentation of the hair (Fuchs, 2008; Fuchs and Horsley, 2008). Once the hair follicle structure is formed, regeneration by initiation of a new hair cycle is achieved by signalling between the stem cells found in the bulge and the secondary hair germ cells (Toyoshima et al., 2012).

The hair follicle undergoes cyclic growth during postnatal life. The hair cycle phases are anagen, a phase of active growth, catagen, which is the regressing phase, telogen, which is the resting stage of the hair cycle, and finally exogen, the hair shedding phase (Hardy, 1992; Milner et al., 2002; Stenn and Paus, 2001). In humans the length of each phase varies and also the duration of each cycle is different depending on the anatomical location of the body (Saitoh et al., 1970). In human scalp, the hair follicle can remain in anagen between two to six years, this is generally the longest period of the cycle. The hair follicle will then regress in the catagen phase which lasts around 2-4 weeks; this stage is generally the shortest stage of the hair cycle. The resting phase will generally be around 1-4 months before the hair follicle is shed and a new anagen phase starts again. Specific mesenchymal-epithelial

interactions control the hair follicle cycle, which involves communication between the specialised fibroblasts of the follicular dermal papilla, the epithelial keratinocytes of the hair matrix and the stem cells of the bulge (Lindner et al., 2000; Stenn et al., 1996).

1.2.4 Hair follicle cycle

1.2.4.1 Anagen

Anagen in the growth phase of the hair cycle (Muller-Rover et al., 2001; Stenn et al., 1996; Stenn and Paus, 2001). It has been widely established that there are two main cell types that are important in hair follicle growth; they are the dermal papilla and the hair follicle stem cells (Hu et al., 2012; Schneider et al., 2009; Yi and Fuchs, 2010). Signalling between the dermal papilla and the hair follicle stem cells initiates the formation of the hair follicle, from the hair matrix to the dermal sheath.

1.2.4.2 Catagen

Catagen is the regression phase and is characterised by the activation of apoptosis and the decrease in cell proliferation and melanogenesis. This involves the cessation of cell growth and pigmentation, the dermal papilla condenses and is released from the bulb, there is loss of the layered differentiation of the lower follicle, remodelling of the extracellular matrix, and apoptosis of the inferior hair follicle, causing the dermal papilla to migrate towards the bulge region (Stenn and Paus, 2001).

In this stage the lower parts of the hair follicle regress; the lower part of the follicle migrates upward to the level of the arrector pili muscle. Apoptosis is a major process that occurs in this phase of the hair cycle, and needs to be tightly regulated (Lindner et al., 1997; Weedon and Strutton, 1981).

1.2.4.3 Telogen

Telogen is the resting phase of the hair cycle. Morphologically, a hair follicle is identified to be in telogen when the hair follicle shaft base has a brush club

feature formed by cornified cells (Milner et al., 2002; Ryder, 1965). Structurally the hair follicle can be divided into two sections, the upper and lower region of the hair follicle, above and below the sebaceous gland. The epidermal layer in the upper region that is a continuation of the skin epidermis has the same mitotic activity in the basal layer; however during telogen, the activity in the lower region is in a state of relative quiescence (Bullough and Laurence, 1956) with little, or only a small amount of DNA and RNA synthesis (Silver and Chase, 1970).

1.2.4.4 Exogen

The specific timing of when this occurs varies between location and hair follicle type (Milner et al., 2002). Studies have shown that this phase of the hair cycle is independent of the other distinct phases (Milner et al., 2002; Stenn, 2005). Previously, it was thought that the shedding of the old fibre from the follicle was due to the new growing fibre dislodging the old (Kligman, 1961). The exact mechanism of how the hair shedding occurs is still unknown (Higgins et al., 2009).

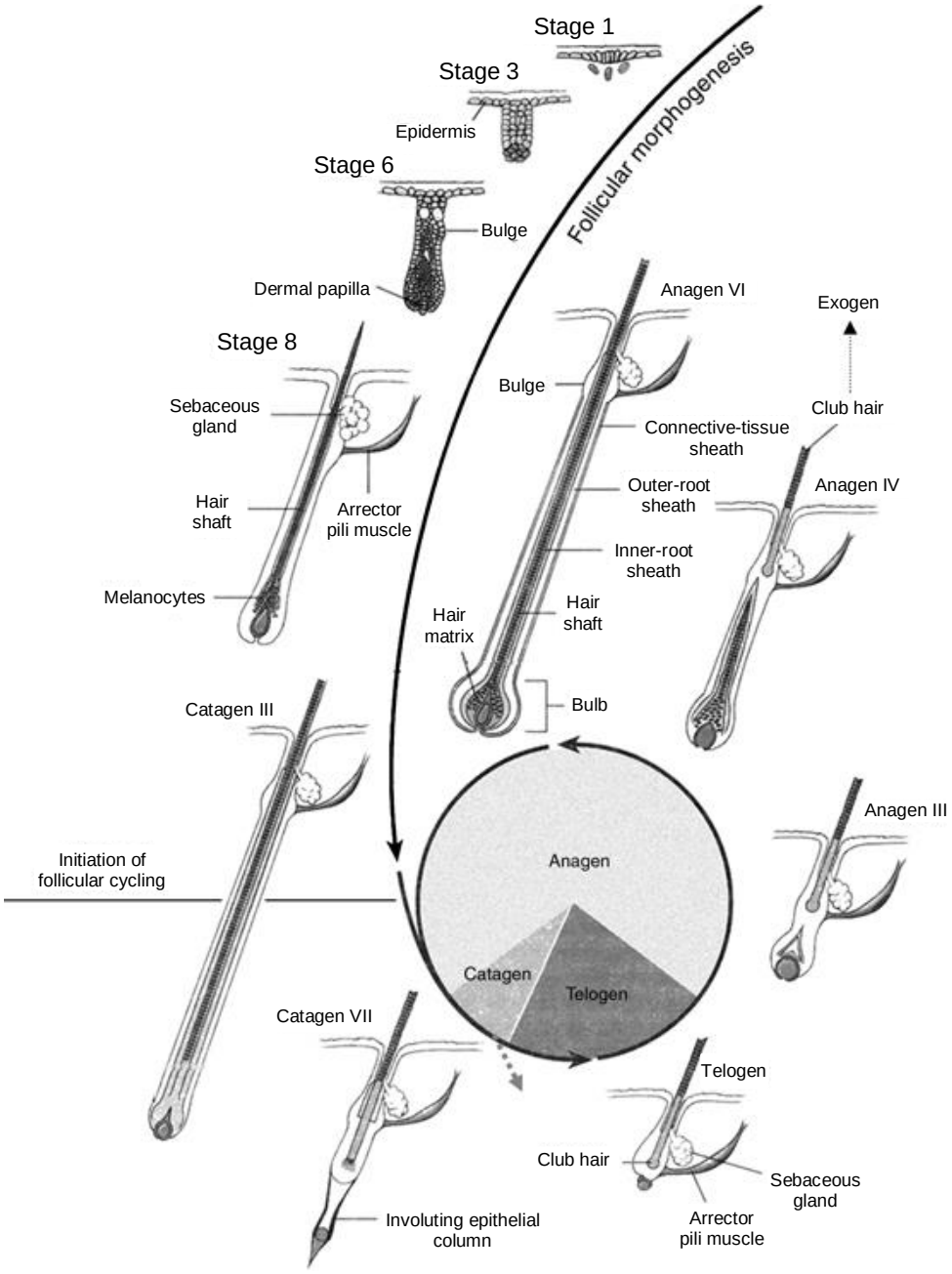


Figure 1.4: Schematic representation of hair follicle morphogenesis and cycling

Mouse hair cycle stages from embryonic development through to the continual cycle (Foitzik et al., 2009).

1.3 Wound Healing

There are four main stages involved in the wound healing process; they are, haemostasis, inflammation, proliferation, and remodelling (Wilgus, 2008).

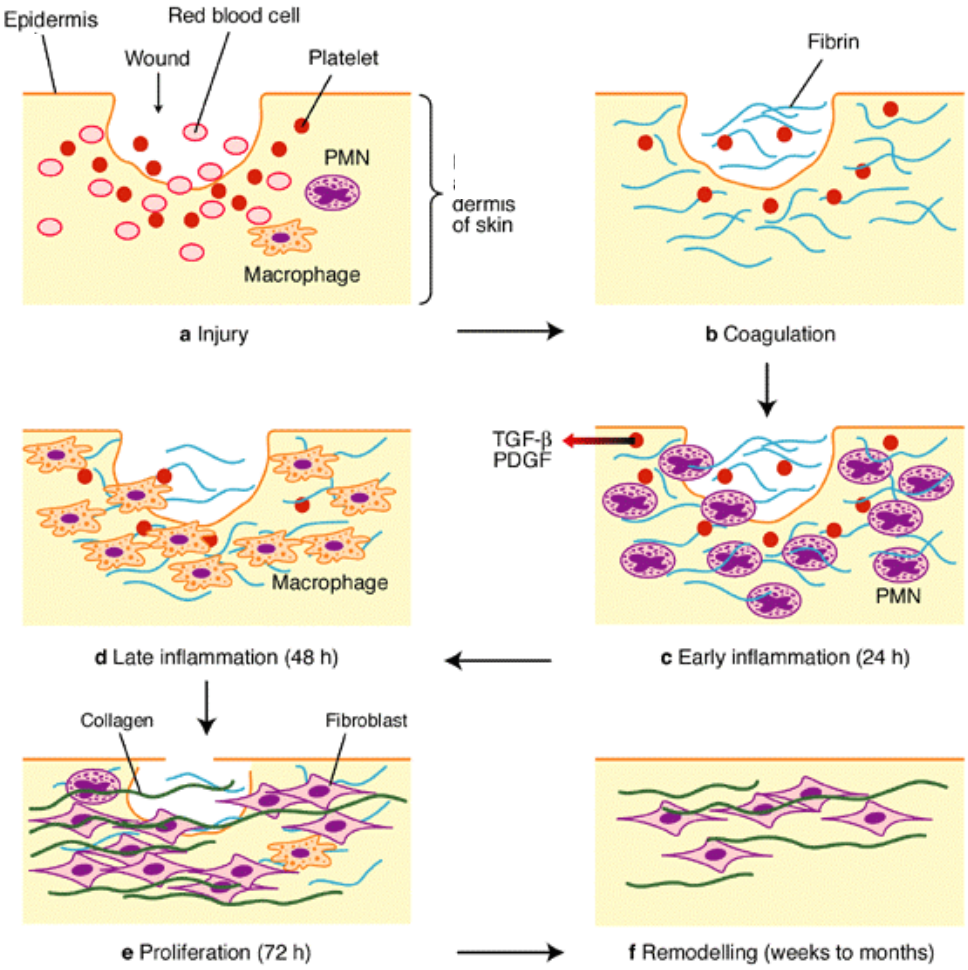


Figure 1.5: Phases of the cutaneous wound healing process.

A flow chart diagram illustrating the phases and events of wound healing. A) At initial wounding, increase in blood cells and platelets at the site of injury, with recruitment of immune cells including macrophages. B) Coagulation occurs to close the wound site; however, platelets also act as a scaffolding for fibroblasts to migrate onto to produce the necessary ECM components required for skin remodelling. C and D) Later, immune cells are further recruited such as neutrophils and macrophages via secretion of cytokines and growth factors. E and F) Myofibroblasts differentiated from fibroblasts cause contraction while fibroblasts continue to produce collagen for re-epithelialisation, building up the new ECM until remodelling is finally finished. TGFβ – transforming growth factor β, PDGF – platelet derived growth factor (Beanes et al., 2003).

1.3.1 Haemostasis phase

This phase of the wound healing process is the initial step, and involves the cessation of blood loss; to ensure that blood pressure is stabilised. The three main process that ensure bleeding stops are: vasoconstriction, platelet activation and coagulation (Fischer et al., 2005b).

The formation of the clot is a temporary solution to the wound repair, protecting the wound from the entry of foreign objects in the environment and also containing the proteins (such as factor V and VIII and fibronectin for coagulation), growth factors (such as platelet derived growth factor (PDGF)) and cytokines (such as interleukin1 (IL1)) required in the wound for repair (Martin, 1997). It also has a role in providing the foundation for which keratinocytes can migrate to rebuild the epidermis (Scheid et al., 2000).

Blood will seep into the wound via the damaged blood vessels in the surrounding area. Platelets then form blood clots protecting the wound, but also act as a mesh to allow cells such as fibroblasts and keratinocytes to migrate through. Once activated platelets degranulate, they release cytokines and growth factors important for wound repair. These growth factors and cytokines provide a chemotactic response recruiting inflammatory cells to the wound site.

1.3.2 Inflammatory phase

There are five important characteristics that indicate that a wound is in the inflammatory phase, they are; redness, warmth, swelling, pain and loss of function, generally associated with the level of pain or muscle spasms (Wild et al., 2010). The growth factors and cytokines produced by platelets at the wound site provide a chemotactic signal that attracts the neutrophils and monocytes to the wound site; however, it is not only the chemotactic signals, but also molecular changes in the endothelial cells that line the capillaries at the wound site that also recruit neutrophils and monocytes (Martin, 1997). Neutrophils are the most abundant inflammatory cells in the early stages of wound healing (Koh and DiPietro, 2011; Ross and Odland, 1968), with the

influx in the number of neutrophils in the wound site monocytes also increase and differentiate into mature macrophages (Koh and DiPietro, 2011; Ross and Odland, 1968), this generally occurs a few days later (Martin and Leibovich, 2005). Neutrophils that enter the wound site mop up the infiltrating bacteria and then are disposed of via phagocytosis by macrophages (Martin, 1997; Singer and Clark, 1999; Wilgus, 2008). Even though the immune response in wound healing is important, not all factors involved are essentially vital for wound regeneration. It has been demonstrated that neutrophils and macrophages contribute to scarring (Wilgus, 2008); however, depletion of certain components of the immune response has been shown to disturb the wound healing process (Martin and Leibovich, 2005; Simpson and Ross, 1972). Certain immune cells such as macrophages are beneficial to wound healing depending on the stage of the wound repair (Mirza et al., 2009); however excessive neutrophils could be detrimental to wound healing, mainly in the re-epithelialisation phase (Wilgus, 2008).

Different growth factors are released in this stage of wound healing such as transforming growth factor β1 (TGFβ1), platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF); these are important for the formation of granulation tissue in preparation for the next stage of the wound healing phase (Singer and Clark, 1999). TGFβ1 is one of the more important growth factors that is central to wound healing (El Gazaerly et al., 2013; Roberts et al., 1986). It has a role in inflammation, recruiting inflammatory cells to the wound site, in the proliferative phase, by influencing the release of collagen into the ECM, and therefore contributing to scar formation (El Gazaerly et al., 2013; Roberts et al., 1986). In a rat study by O'Kane and Ferguson (1997), they demonstrated that in a wound healing response there are two peaks in TGFβ1 levels. The first was seen 1 hour post-wounding, while the second peak was seen 7 days post wounding. This could be attributed to the different stages of the wound healing process.

1.3.3 Proliferative phase

Also known as granulation tissue formation, this phase is characterised by angiogenesis, myofibroblast differentiation and fibroplasias (Amadeu et al., 2008; Hinz, 2007; Singer and Clark, 1999). This stage of wound healing can be split into stages; they are re-epithelialisation, granular tissue formation, neovascularisation and wound contraction.

1.3.3.1 Re-epithelialisation

Initially it was thought that the platelets that are first seen at wound sites produce the necessary growth factors that would help at later stages of wound healing. However, a study by Szpaderska et al. (2003) in mice demonstrated that in the absence of platelets, re-epithelialisation, collagen synthesis and angiogenesis still occurred at a normal rate, indicating that even though platelets are important for the initial stages of wound healing, they do not contribute to the proliferative phase. In regards to the inflammatory phase, macrophages and T cells were increased but no difference was seen in neutrophil levels compared to control mice. This study also looked at the levels of VEGF, FGF2, TGFβ1, KGF and EGF and noted that there were no significant differences between mice lacking platelet function and controls. In contrast, the increase in neutrophils in the inflammatory response, caused a negative impact on wounding in mice; a depletion in neutrophils increased the rate of re-epithelialisation (Dovi et al., 2003).

An important aspect of re-epithelialisation is the ability to produce the cells (keratinocytes and fibroblasts) required for the foundation of the wound repair. This involves the stem cells located in the skin epidermis, and in some cases where hair follicles are present, the stem cells in the bulge of the hair follicle. Generally, during wound healing, it was thought that stem cells from the basal layer of the epidermis were used to regenerate the damaged epidermis; however with new markers that distinguish between the two different stem cell niches, it is becoming apparent that stem cells from the hair follicle bulge also contribute to epidermal reconstruction during wounding (Ito and Cotsarelis,

2008; Ito et al., 2005; Plikus et al., 2012). It has been shown in mice that without the bulge stem cell niche, the skin is capable of wound repair, however, this niche allows for a faster rate of epithelialisation (Ito et al., 2005). Also, cells from the bulge do not contribute to the closure of the wound. Approximately 25% of re-epithelialised cells in the epidermis where hair follicles are found are derived from the bulge (Ito and Cotsarelis, 2008). Interestingly, these cells are temporary, in that once they migrate into the epidermis, they lose their follicular markers, but express epidermal differentiation markers (Ito et al., 2005). Bulge-derived cells are not long lasting, and reduce in number after 20 days of wounding. The initial percentage of bulge-derived cells constitutes 26% of re-epithelialised cells, and is reduced to 3.5% after 20 days; whilst non-bulge derived cells persist in re-epithelialisation (Ito et al., 2005; Levy et al., 2005). The contribution of bulge stem cells to epidermal regeneration is only wound responsive. A contributing factor with regards to the link between hair follicles and wound healing is the stage in which the hair follicle is in during the hair cycle (Ansell et al., 2011). In this mouse study, it was demonstrated that wound healing was accelerated (wound area was reduced) when hair follicles were in the anagen phase of the hair cycle; there was an increase in angiogenesis and accelerated ECM deposition.

There have been no studies looking at the impact of vellus hair follicles on wound healing, many studies are conducted on animal models mainly mice which do not possess vellus hair follicles (Driskell et al., 2009). Due to the size of vellus and terminal hair follicles and the location of the bulge relative to the epidermis, wound healing responses may differ (Garcia et al., 2011).

1.3.3.2 Neovascularisation

Another important stage of the wound healing process is neovascularisation; this involves angiogenesis which helps not only in providing oxygen and nutrients to the healing tissue, but in the recruitment of immune cells such as neutrophils and macrophages to the damaged site. Its response is dependent on the pro-angiogenic factors released from infiltrating macrophages and

keratinocytes; these factors include VEGF, FGF and angiopoietin1 (Greaves et al., 2013).

1.3.3.3 Granular tissue formation

Tissue granulation typically begins around 4-6 days after initial wounding. It is the production of new connective tissue by fibroblasts. Important signalling molecules such as transforming growth factor β1 (TGFβ1) and bone morphogenetic proteins (BMPs) are important factors that regulate growth, differentiation and matrix synthesis, and are required for wound healing (Jaffe et al., 2012).

An important factor in this process is the ability of fibroblasts to differentiate into myofibroblasts which are important in migration and contraction, this is via the upregulation of α smooth muscle actin (αSMA) (Hinz, 2007; Hinz et al., 2007). One of the main growth factors that stimulates this differentiation is TGFβ1 (Midgley et al., 2013). Myofibroblasts are not only required for contraction and migration, but also in cell-to-matrix attachment and intracellular adhesion (Hinz, 2007). There are potentially other factors such as the pro-inflammatory cytokine IL6 secreted from macrophages, that also contributes to fibroblast differentiation (Gallucci et al., 2006).

Fibroblasts are also responsible for scar formation, with wounding, fibroblasts are activated to release collagen into the wound site to help rebuild the ECM, but excess collagen and its irregular distribution can result in scarring (Darby and Hewitson, 2007; McDougall et al., 2006).

During cutaneous wound healing, dermal fibroblasts migrate into the wounded area, secreting large amounts of collagenous matrix reforming the dermis, and providing ECM scaffolds for keratinocytes (Alberts, 2002). There are many factors that influence dermal fibroblasts to migrate during wound healing; one of these factors are hormonal effects. Oestrogen has been shown to significantly increase migration of human dermal fibroblasts cultured from female breast skin *in vitro* (Stevenson et al., 2005).

Dermal fibroblasts also produce soluble factors such as keratinocyte growth factor 1 (KGF1) a member of the fibroblast growth factor (FGF) family, which helps regulate wound repair via keratinocyte proliferation (Sorrell and Caplan, 2004). An *in vitro* study by Moulin et al. (2000) compared the effect of myofibroblasts and fibroblasts extracted from breast skin during wound healing on keratinocyte re-epithelialisation. During wound healing, myofibroblasts did not interact with keratinocytes to support keratinocyte differentiation and basement membrane formation in comparison to normal dermal fibroblasts (Moulin et al., 2000). In a comparison of site matched papillary and reticular fibroblasts from breast skin *in vitro*, the production of KGF1 was higher in reticular fibroblasts compared to papillary fibroblasts indicating that reticular fibroblasts play a more dominant role in keratinocyte differentiation, important in wound healing (Sorrell et al., 2004).

Collagen synthesis in response to TGFβ1 is not stimulated in patients with venous ulcers, this is due to the reduced TGF β type II receptor, affecting wound healing. A study by Hasan et al. (1997) showed that in human *in vitro* studies, the inability of dermal fibroblasts cultured from biopsies taken from patients with venous ulcers at the edge of the wound, to respond to TGFβ1 caused faulty deposition of ECM affecting re-epithelialisation and adversely wound healing (Hasan et al., 1997). *In vitro* studies have shown that fibroblasts respond differently to cytokines depending on the condition in which these cells were cultured; the mitogenic potential of fibroblasts cultured from diabetic ulcers was reduced when stimulated by different cytokines such as IGF1, FGF, EGF and PDGF (Loots et al., 2002).

1.3.3.4 Wound contraction

The final stage of the proliferative phase of wound healing is wound contraction. One of the most important elements in this stage is the differentiation of dermal fibroblasts into the myofibroblast, which are generated early in wound healing and contribute throughout the process. It is these cells that are responsible for contraction due to their smooth muscle property (Sorrell and Caplan, 2004). The combination of both fibroblasts and

myofibroblasts improve wound healing with fibroblasts providing traction whilst myofibroblasts provide contraction (Li and Wang, 2011). The myofibroblasts later disappear via apoptosis and are replaced by dermal fibroblasts that help produce the collagen and build up the ECM (Desmouliere et al., 1995; Sorrell and Caplan, 2004). Excess contraction and ECM deposition leads to scarring, therefore it is important to regulate both contraction and collagen synthesis (Li and Wang, 2011). A number of factors can modulate fibroblast contractility; tensin, a protein found in the plasma membrane has been shown to modulate fibroblast contractibility and affect ECM remodelling in wound healing (Clark et al., 2010); particularly the tensin 2 isoform, its knockdown in cultured human foreskin fibroblasts inhibited contraction. Epidermal growth factor (EGF) also influences fibroblast contractility (Iwabu et al., 2004). Stimulation of EGF increases the phosphorylation of myosin light chain, a marker for contractile force. Also TGFβ1 secretion correlates with fibroblast contractility (Coleman et al., 1998). These studies used collagen gels to analyse fibroblast contraction. Studies have been conducted in cultured dermal fibroblasts derived from female scalp showing that collagen synthesis is increased with mechanical wounding and that there is an increase in VEGF and TGFβ1 secretion (Stevenson et al., 2008b). There are limited studies directly comparing dermal fibroblast migration and collagen synthesis by cells cultured from different regions of the same donor.

1.3.4 Remodelling phase

This phase of wound healing involves collagen remodelling and angiogenesis (Guo and Dipietro, 2010). This stage of the wound healing process may last for up to two years. Neovascularisation occurs during wound healing to bring nutrients and other factors that improve the quality of wound repair. However, once this event has finished, myofibroblasts that are present and the new vascular structure need to be removed from the site. The regression of the vascular structure is done via apoptosis, while the myofibroblasts are removed by one of three ways; necrosis, migration or apoptosis (Greenhalgh, 1998).

Another important feature is the remodelling of the ECM to represent that of normal tissue (Campos et al., 2008; Gosain and DiPietro, 2004).

1.3.5 Contribution of the hair follicle to wound healing

Wounds heal better in skin bearing terminal hair follicles as opposed to those that do not (Jahoda and Reynolds, 2001). This has been hypothesised to be not only due to the hair follicle bulge that contains a niche of stem cells important for wound healing, but also due to the follicular dermal sheath and dermal papilla. It is thought that the dermal sheath fibroblasts act as progenitors for interfollicular dermal fibroblasts, thereby improving wound healing (Jahoda and Reynolds, 2001). Another study showed that in rodents, the number of fibroblasts that expressed αSMA from different regions of highly dense hair follicle skin, and skin containing intermediate hair follicles and skin containing no hair follicles varied in expression between young and old rodents (Reynolds et al., 1993). The expression of αSMA was highest in skin containing no hair follicles in young 4 day old rodents, and lowest in highly dense terminal hair follicle skin, the opposite results were seen in adult rodents. This has been attributed to hair follicles; with emphasis on the hair cycle influencing wound healing; in 4 day old rodents, the hair follicle is in the initial morphogenesis stage of the hair cycle, whilst in adults, hair follicles are more likely to be in anagen. In this study, the hair follicle stage was not mentioned for both age groups. Studies on rodents showed that wounds heal faster when the hair follicle is in the active growth phase anagen, in comparison to when the hair follicle is in the regressive catagen or resting telogen phase (Ansell et al., 2011; Jahoda and Reynolds, 2001; Zawacki and Jones, 1967).

Apoptosis is a process that is tightly controlled which in turn controls the number of cells present at individual stages of the wound healing process. It has been shown to occur in the early stages of wound healing with inflammatory cells (Brown et al., 1997). During the proliferative phase and granulation phase, apoptosis occurs to maintain myofibroblast numbers and coordinate the removal of these cells once they are no longer required

(Desmouliere et al., 1995). Therefore, it is an important pathway to investigate to further understand how the number of these cells that are important for wound healing are maintained and how wound healing is affected.

1.4 Apoptosis

Apoptosis is a physiological process of programmed cell death. It is characterised by changes to the cell, both morphologically and biochemically including cell shrinkage, nuclear chromatin condensation, DNA fragmentation, and proteolysis of caspases (Shi, 2002; Thornberry, 1997). Apoptosis plays a role in many different aspects of cell growth and development. It occurs in response to homeostatic changes, maintaining the number of cell turnover; allowing for growth of new cells and the cause of death of old and damaged cells. This specific process also plays a role in the immune defence in humans by disposing of dead cells in the organism (Wei et al., 2008). There are many different pathways that are involved in apoptosis, each involving different proteins to regulate the process, and each one linking with one another to make a more structured and definitive pathway. The two main pathways of apoptosis are the intrinsic and the extrinsic pathways, where the intrinsic pathway involves the mitochondria, whilst the extrinsic pathway involves cell surface death receptors (Wei et al., 2008).

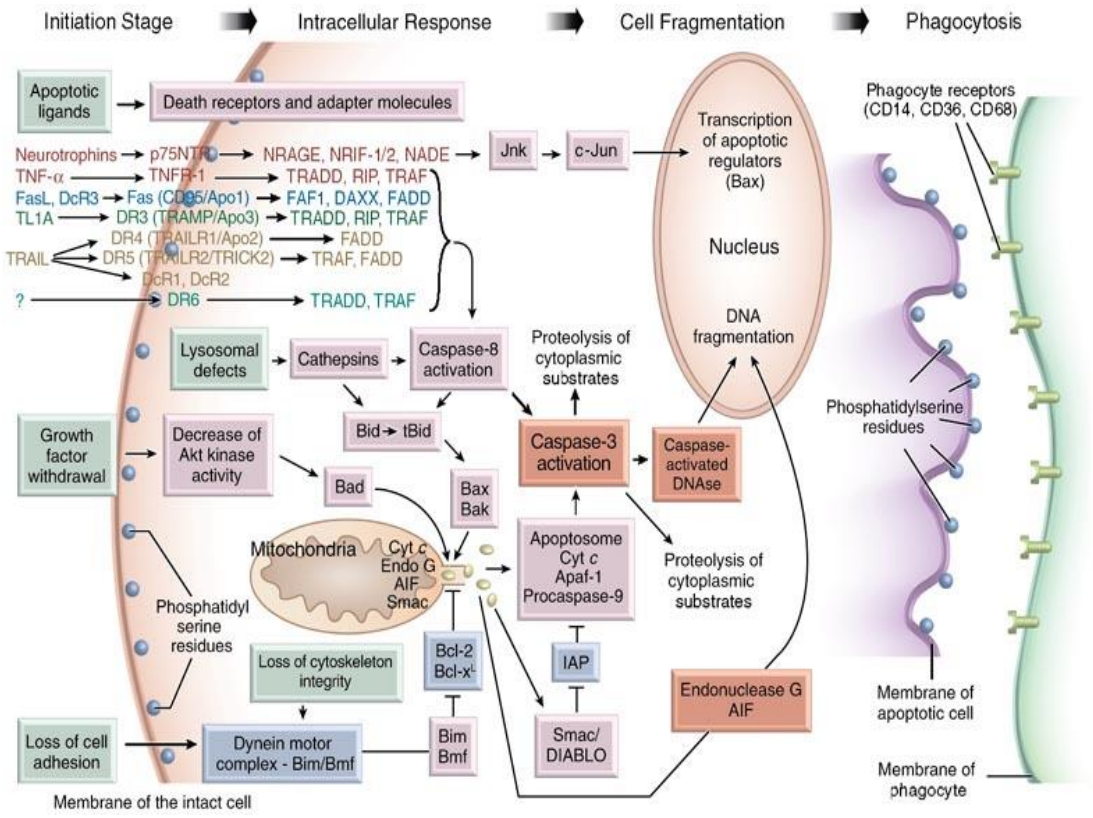


Figure 1.6: Schematic representation of the apoptosis pathway

The four stages of apoptosis initiation, intracellular response, cell fragmentation and phagocytosis, which are highlighted in the diagram representing a cell death with different signalling molecules activated in the different stages. The two main apoptotic pathways are illustrated with the extrinsic pathway being activated from the cell receptors, and the intrinsic pathway involving the mitochondria leading to the common pathway activating the caspases, AIF – apoptosis inducing factor, IAP – inhibitor of apoptosis protein (Botchkareva et al., 2006).

There are four main phases involved in apoptosis; the initiation stage, intracellular response, cell fragmentation and eventually phagocytosis (Figure 1.6). The initiation stage of apoptosis involves the detection of different stimuli at the cell surface, this may include different apoptotic ligands, or changes in growth factor levels; and involves the activation of the extrinsic pathway. Environmental changes can also trigger apoptosis without the use of cell surface receptors; these include free radicals, UV radiation, and drugs

such as those used in chemotherapy (Buttke and Sandstrom, 1994; Schwall et al., 1993; Yamada and Ohyama, 1988).

The detection of these changes causes an intracellular response, which is the second stage of apoptosis, where different proteins that regulate apoptosis interact and are released into the cytosol. An important family of proteins that regulate apoptosis are the caspases. The caspase family members are very important in apoptosis and its regulation as caspases modulate the activation of transcription factors (Hofmann et al., 1997), which are important in the regulation of pro- and anti- apoptotic proteins, which in-turn mediate cell fate. Procaspase-8 is able to detect the receptor-ligand complex initiating the caspase cascade forcing the cell into cell death (Boldin et al., 1996). Once the cell has reached this stage of apoptosis, the fate of the cell is determined and is irreversible; this applies to both the intrinsic and extrinsic pathways of apoptosis. Although caspases have an important role in apoptosis, apoptosis may still occur independently of caspase activity via the activation of Apoptosis Inducing Factor (AIF) (Susin et al., 1999). This protein is found in the mitochondrial intermembrane space and once released, is translocated into the nucleus where it binds to the DNA causing chromatin condensation (Cande et al., 2002).

The third stage of apoptosis is cell fragmentation, where morphological changes can be observed, including cell shrinkage, although the plasma membrane is still intact. Chromatin condenses and migrates to the nuclear membrane periphery; there is also an increase in mitochondrial activity (Green and Reed, 1998). An important step in this stage is the exposure on the cell surface of anionic phospholipids and phosphatidylserine, these are signalling molecules that are recognised by phagocytes (Savill, 1997). At the end of this stage, the membrane encapsulates organelles in the cell, resulting in the presence of many small vesicles known as apoptotic bodies.

The final stage of apoptosis is the phagocytosis of the apoptotic body by phagocytes; which are capable of recognising the apoptotic bodies due to the receptors found on their surface that detect the exposed phosphatidylserine

residues and other molecules at the surface of the apoptotic bodies. Failure to recognise the apoptotic cells would lead to the persistence of inflammation, eventually assuming necrotic morphology known as secondary necrosis (Afford and Randhawa, 2000).

During wound healing, depending on the type of wound, cells will either undergo necrosis or apoptosis. In chronic wounds where inflammation is present at a high level, cells generally go through necrosis. However, in acute wounds where necrosis and inflammation are not continually high, cell number decreases either via cells migrating away from the wound site or apoptosis (Greenhalgh, 1998). The types of cells that would undergo this process are the inflammatory cells once they have completed their roles, and also fibroblasts once enough collagen has been deposited. Studies in mice have shown that apoptosis is present at early stages of wound healing in the granular tissue where high levels of inflammatory cells are still present, with no apoptosis detected in the granular tissue at the wound edge where fibroblasts are migrating (Brown et al., 1997; Desmouliere et al., 1997). Another study in rats showed that myofibroblasts underwent apoptosis during wound closure (Desmouliere et al., 1995). The importance of apoptosis is seen when there is a delay in signalling for the down regulation of the activity of both fibroblasts and myofibroblasts beyond a time point, apoptosis is not activated leading to excessive collagen synthesis and scar formation (Greenhalgh, 1998).

1.4.1 Intrinsic pathway

The regulatory point in the intrinsic pathway in which apoptosis occurs is in the mitochondria (Festjens et al., 2004; Wong and Puthalakath, 2008). It is at the outer mitochondrial membrane, endoplasmic reticulum and cytoplasmic aspect of the nuclear envelope that the pro- and anti- apoptotic proteins reside, and contribute to the decision whether the cell should live or die (Krajewski et al., 1994; Lithgow et al., 1994; Wong and Puthalakath, 2008). The intrinsic pathway is activated by different stress stimuli which causes alterations in the levels of Bcl-2 leading to the release of cytochrome c and AIF from the

mitochondrial intramembrane into the cytosol (Daugas et al., 2000). The permeability of the mitochondria to release cytochrome c and AIF are tightly regulated by the Bcl-2 super-family (Table 1.3), including both pro- and anti-apoptotic proteins (Adams and Cory, 1998; Gross et al., 1999; Strasser et al., 1995).

Bcl-2 Super-family		
Pro-Apoptotic		**Anti-Apoptotic**
Bik	Bax	Bcl-2
Bad	Bak	Bcl-X_L
Bim	Bok	Bcl-w
Hrk	Boo	Bcl-1
Bcl-G	Bcl-B	Bcl-B
Noxa	Bcl-rambo	A1
Puma	Bid	Mcl-1
Bcl-X_S		Boo

Table 1.3: Bcl-2 Family members divided into pro- and anti- apoptotic proteins

Pro- and anti- apoptotic proteins of the Bcl2 family members, Bcl2 – B cell lymphoma 2, Bik – Bcl2 interacting killer, Bad – Bcl2 associated death promoter, Bim – Bcl2 like protein 11, Hrk – activator of apoptosis hara-kiri, Noxa – Phorbol12myristate13acetate induced protein 1, Puma – p53 upregulated modulator of apoptosis, Bcl Xs – b cell lymphoma extra small, Bcl Xl – b cell lymphoma extra large, Bax – Bcl2 associated X protein, Bak – Bcl2 homologous antagonist killer, Bok – Bcl2 related ovarian killer, Bid – BH_3 interacting domain death agonist Bcl w – Bcl2 like protein 2, A1 – Bcl2 related protein A1, Mcl1 – induced myeloid leukaemia cell differentiation protein Mcl1 (Mayer and Oberbauer, 2003; van Gurp et al., 2003).

The most important proteins from the Bcl-2 super-family are Bcl-2 and Bax, these proteins inhibit or promote, respectively, the permeability of the mitochondrial membrane (Reed, 2006). AIF has the capability of mediating a caspase-independent mitochondrial apoptosis pathway by moving to the nucleus and causing chromatin condensation and DNA fragmentation

(Daugas et al., 2000). Cytochrome c binds to apoptotic protease activating factor 1 (Apaf1) with the use of energy in the form of dATP to form a protein complex named apoptosome, capable of promoting the autoactivation of procaspase-9 (Sun et al., 1999).

1.4.2 Extrinsic pathway

The extrinsic pathway involves death domain containing receptors found on the cell surface, these include Fas and tumour necrosis factor receptor 1 (TNFR1); these receptors are members of the TNF super-family. Their primary amino acid structure is slightly different, but they all share the same cysteine-rich extracellular subdomain giving rise to a similar tertiary structure (Mayer and Oberbauer, 2003). Once these receptors are activated by interacting with a corresponding ligand, they form a multimeric complex (Lavrik et al., 2005) generally by the death domain of the adapter molecules such as Fas-Associated protein with Death Domain (FADD) and TNF receptor-associated death domain (TRADD) (Mayer and Oberbauer, 2003). TRADD is able to recruit both TNF Receptor Associated Factor 2 (TRAF2) and Really Interesting Protein (RIP) via its amino-terminal region and its death domain (Mayer and Oberbauer, 2003).

These molecules are needed for the formation of the TNFR1 signalling complex (Park et al., 2007), without the TNFR1 signalling complex, NFκβ is not activated, which is an important transcription factor; however, with the suppression of NFκβ, TNFα is able to promote apoptosis via the formation of a secondary signalling complex (Varfolomeev et al., 2006). TRADD, TRAF2 and RIP are then translocated into the cytosol, where they recruit FADD and caspase 8 and 10 forming a death-inducing signalling complex (DISC), which has the capability of upregulating the cascade signals that activate capase-8 (Wilson, 1998). Once procaspase-8 is activated due to its positioning in the DISC (Boatright et al., 2003), caspase 8 is released into the cytosol.

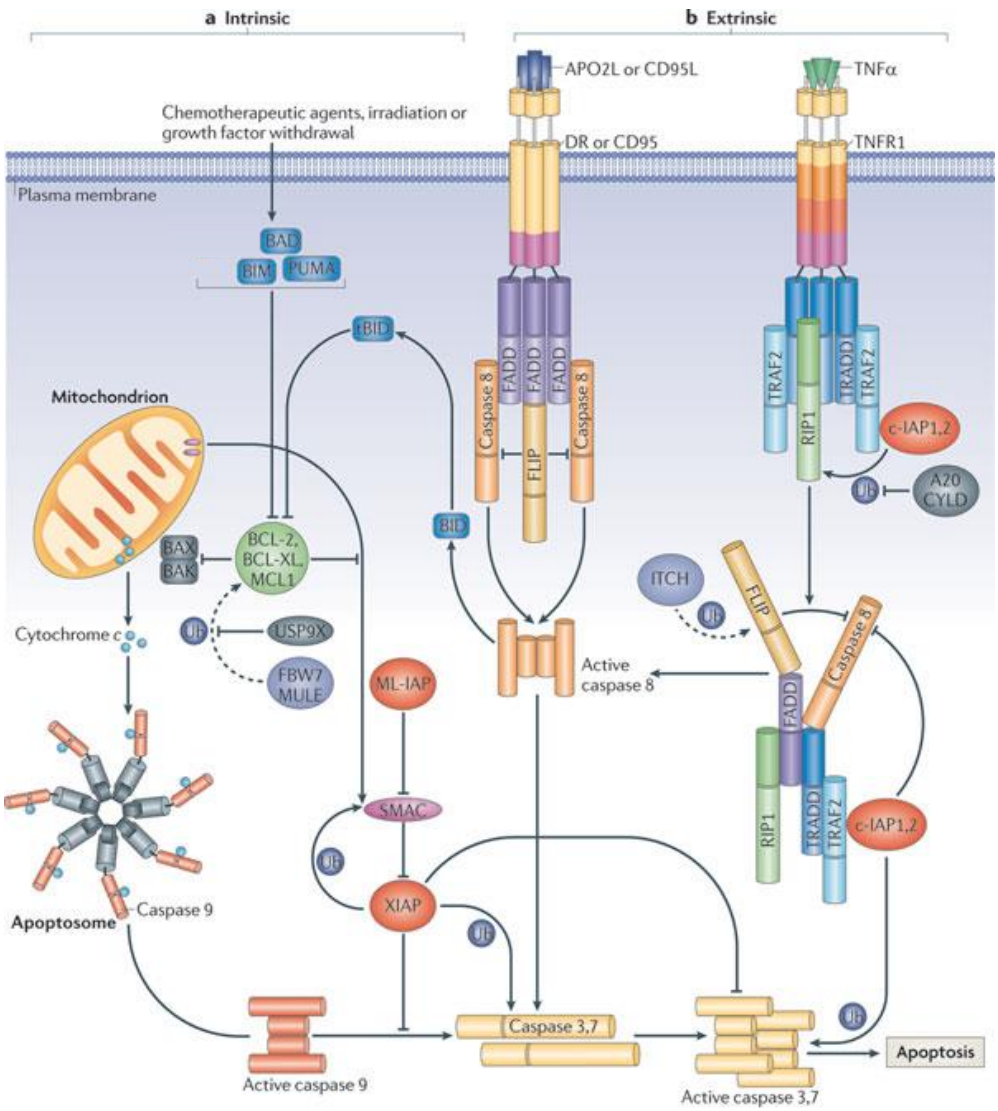


Figure 1.7: Schematic representation of the intrinsic and extrinsic apoptosis pathway

The intrinsic (via mitochondrial pathway) and extrinsic (via death receptors) pathway to eventually activate the caspase cascade which leads to cell death. BAD – Bcl2 antagonist of cell death, BIM – Bcl2 mediator of cell death, PUMA – p53 upregulated modulator of apoptosis, TRADD – TNFR1 associated death domain, FADD – Fas associated death domain, FLIP – FLICE inhibitory protein, ITCH – itchy E3 ubiquitin protein ligase (Vucic et al., 2011).

1.4.3 Caspase cascade

Caspases are cytosolic cysteine containing proteasomes, that, the moment they are activated, cause a cascade of events that are irreversible, eventually

leading to cell death (Cryns and Yuan, 1998; Thornberry and Lazebnik, 1998). There are 14 caspase molecules that have been identified in mammals, however, not all the caspase molecules are involved in apoptosis, there are some that are involved in skin cell development; a study by Fischer et al. (2005a) studied both caspase 3 and caspase 14 in keratinocyte differentiation. Increased levels of caspase 14 were seen in mice at embryonic day 14 and onwards, a time coinciding with skin development and keratinocyte differentiation (Fischer et al., 2005a). Other caspases have been shown to have other roles, for example, caspase 1, -4, -5 and -11 are implicated in activation of pro-inflammatory cytokines, interleukin 1 and 18 (Martinon and Tschopp, 2004). There are two types of caspase molecules, they are the initiator caspases, which can autoubiquitinate, activating the caspase molecule, and the executioner caspases, which are activated by the initiator caspases and cleave other caspase molecules activating them (Fadeel and Orrenius, 2005).

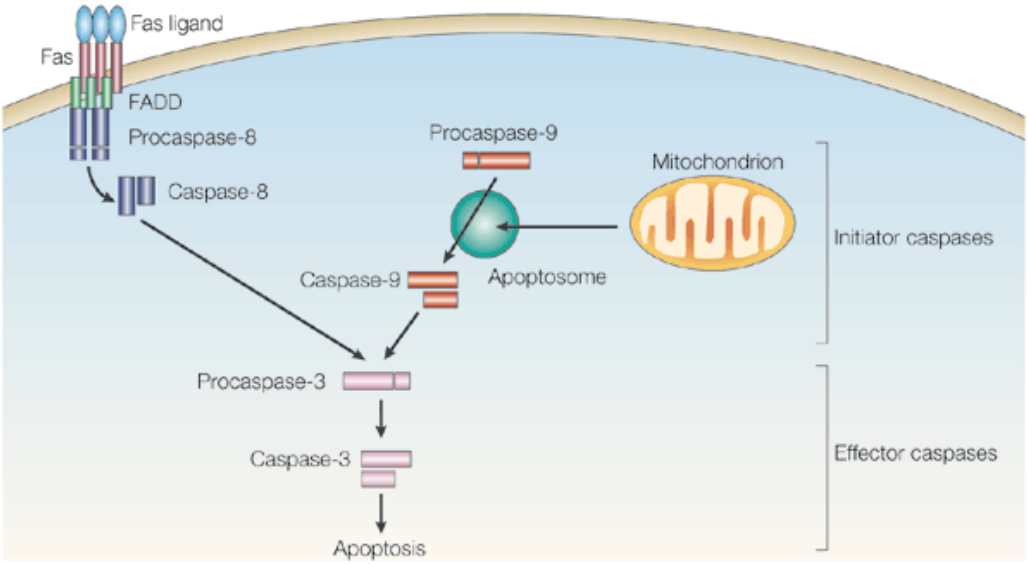


Figure 1.8: Schematic representation of the caspase cascade

The initiator caspases (caspase-8 and -9) and the effector caspase (casase-3). The initiator caspase can be activated via the intrinsic or extrinsic apoptosis pathway. When the inactive pro-caspases are cleaved, they are able to cleave other pro-caspases activating them eventually leading to cell death (Holcik and Korneluk, 2001).

The active forms of the caspase both from the intrinsic (caspase-9) and extrinsic (caspase-8) pathways activate caspase effectors, caspases -3, -6 and -7 which then leads to apoptosis via the cleavage of proteins (Strasser et al., 2000). For clear catalytic activity to be seen, it is important that caspase-9 is bound to the Apaf-1 complex through tight interactions between their Caspase Recruitment Domain (CARD) (Qin et al., 1999; Rodriguez and Lazebnik, 1999). The method by which the complex is able to catalyse other pro-caspase-9 proteins was studied by Malladi et al. (2009). Two hypotheses were identified, either via a displacement model system, or through similar catalytic processes to pro-caspase-3 (Malladi et al., 2009). In this study, it was demonstrated that procaspase-9 is not processed like procaspase-3 as a normal substrate. Instead, procaspase-9 is catalysed through its recruitment into the Apaf-1 complex and displaced continuing the cycle. Caspase-2 has been shown to become activated by stress stimulated cytokines; suggesting that the intrinsic pathway is an amplification of the caspase cascade (Lassus et al., 2002). At the end of the caspase cascade activation phase, caspase-3 is cleaved which causes DNA fragmentation via a DNA fragmentation factor (Mayer and Oberbauer, 2003).

There have been a number of studies investigating the role of caspases in wound healing. It is known that apoptosis is part of the wound healing process, and it would be important and beneficial to understand which caspases are involved in this mechanism. A study by Lee et al. (2009) looked at the levels and effects of caspase 8 in the epidermis of mice during a wound healing response. Fluctuation was seen in the epidermis close to the wound site after 14 days of wounding. Knockout of caspase 8 correlated with an increased level of keratinocyte differentiation and expression of β4 integrin; these observations led to the belief that the loss of caspase 8 causes a paracrine signalling of interleukin 1α to the stem cells to proliferate (Lee et al., 2009). Another study in mice, measured the levels of caspase 3 and -7 via Western blotting as these are effector caspases at the end process in the caspase cascade (Carter et al., 2009). In this study, levels of procaspase 3 and cleaved

caspase in mice was not altered after wounding; however there was an increase in cleaved caspase 7 after wounding.

1.5 Inhibitors of Apoptosis Proteins (IAPs)

As the name suggests, inhibitors of apoptosis proteins (IAPs) are proteins that have the ability to prevent apoptosis. These IAPs were identified whilst investigating the pathways that might regulate apoptosis (Deveraux and Reed, 1999; Nachmias et al., 2004; Yang and Li, 2000; Zhang et al., 2004). The first IAP was identified from *Cydia pomonella* granulosis virus (Crook et al., 1993). In 1995, came the discovery of the first IAP in mammals. In total eight IAPs have been identified in humans (Table 1.4); they are neuronal apoptosis inhibitory protein (NAIP) 1 and 2, cellular IAP (cIAP)1, cIAP2, X-chromosome linked IAP (XIAP), melanoma IAP (ML-IAP), Apollon, Survivin and IAP like protein (ILP)2 (Hunter et al., 2007; Salvesen and Duckett, 2002).

IAP Abbreviations	Full Name	Tissue Expression	Reference
XIAP/ILP-1/MIHA/BIRC4	X-linked IAP	Most human tissue	Duckett et al. (1996)
cIAP1/hIAP1/MIHB/BIRC2	Cellular IAP1	Most human tissue; highly expressed in adult thymus, testis, ovary	Rothe et al. (1995)
cIAP2/hIAP2/MIHC/BIRC3	Cellular IAP2	Most human tissue; highly expressed in adult spleen, thymus	Rothe et al. (1995)
NAIP/BIRC1	Neuronal apoptosis inhibitory protein	Adult liver, placenta, central nervous system	Roy et al. (1995)
Apollon/BRUCE/BIRC6	Apollon	Golgi complex and the vesicles	Hauser et al. (1998)
ILP2/TS-IAP/BIRC8	IAP-like protein 2	Adult testis	Richter et al. (2001)
ML-IAP/Livin/KIAP/BIRC7	Melanoma IAP	Foetal liver, kidney; adult testis, thymus	Vucic et al. (2000)
Survivin/TIAP/BIRC5	Survivin	Foetal liver, kidney, lung, gastrointestinal tract; highly expressed in tumour	Ambrosini et al. (1997)

Table 1.4: Mammalian IAP family members

The IAPs identified in mammalian tissue adapted from Wei et al. (2008).

In addition, four IAP antagonists have been identified in humans; XIAP associated factor 1 (Xaf1) (Liston et al., 2001), second mitochondria derived activator of caspase/ direct IAP binding protein with low pl (Smac/DIABLO) (Du et al., 2000), high temperature requirement protein A2 (HtrA2) (Faccio et al., 2000) and apoptosis related protein in the TGFβ signalling pathway (Arts) (Larisch et al., 2000).

IAPs not only interact with caspase proteins inhibiting their activity (Deveraux et al., 1998), but they also promote proteasomal degradation by catalysing ubiquitination (Qiu and Goldberg, 2005). IAPs are expressed at different levels in cells of the same lineage located in different organs of the human body; it has been proposed that the maturation state of the cell affects the expression levels of the IAPs (Hasegawa et al., 2003; Vischioni et al., 2006).

It is important that IAP levels are regulated to maintain the balance of homeostasis between cell death and cell survival. There are different ways in which IAPs levels are controlled, one is via transcriptional regulators including NF-κβ (Stehlik et al., 1998; Wang et al., 1998), hypoxia inducible factor 1 (HIF1) (Dong et al., 2001), and via post-translational modification, via the degradation of proteasomes (Yang et al., 2000). The Jun amino terminal kinase (JNK) signalling cascade, part of the mitogen activated protein kinase (MAPK), a transcription regulator, is activated. This is via the binding of specific IAPs such as XIAP, NAIP and Ml-IAP to the JNK regulator transforming growth factor β activated kinase 1 (TAK1). Binding of IAPs to TAK1 allows cells to survive against TNFα and caspase-1 induced apoptosis(Figure 1.9).

Whilst studying different types of IAPs, one study led to the belief that IAP expression might be controlled by both cell type-dependant, and organ-dependant regulatory mechanisms (Vischioni et al., 2006). This was due to an observation made between levels of cIAP1, cIAP2 and XIAP. Both cIAP1 and cIAP2 are expressed in cells originating from epithelial and mesenchymal origin. Expression of XIAP was more limited to certain cell types, and not abundantly expressed in the same manner as cIAP1 and cIAP2 (Vischioni et al., 2006).

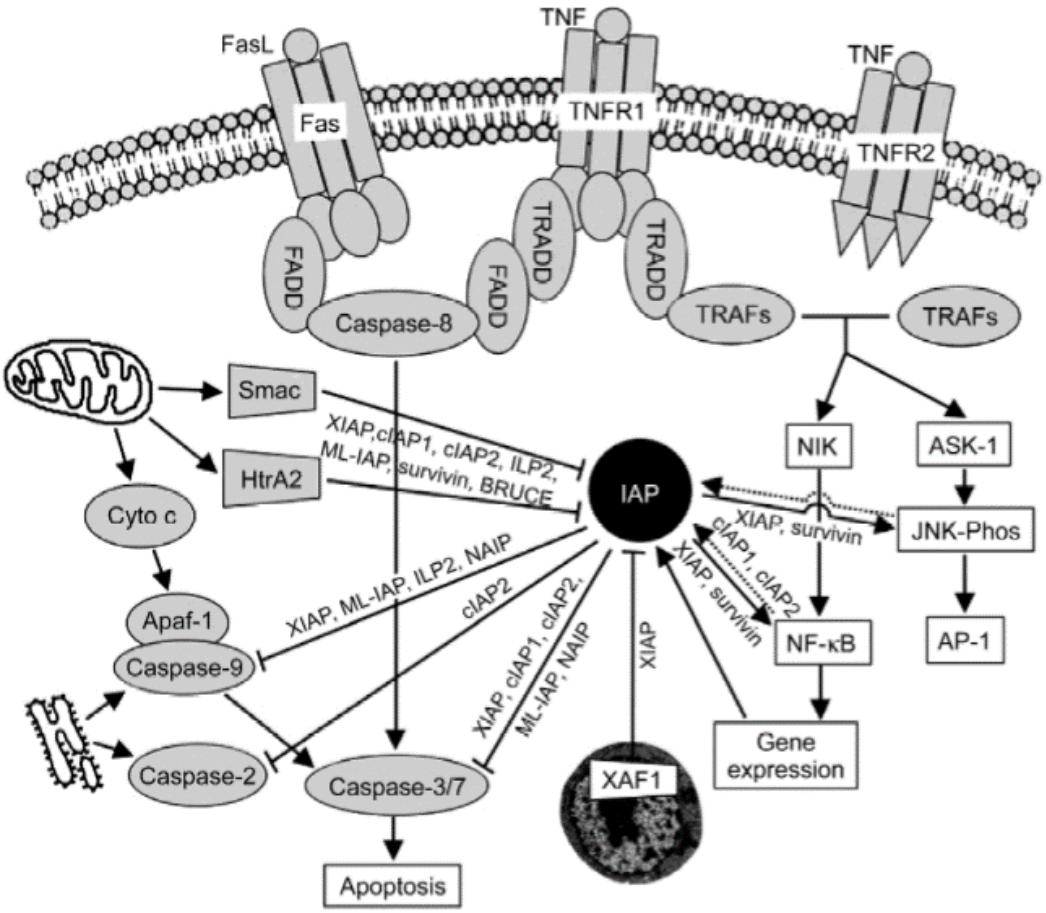


Figure 1.9: IAP and IAP antagonist mechanism of action

The mechanism of action for the different IAPs and IAP antagonists during apoptosis. Intrinsic or extrinsic stimuli can trigger apoptosis, activation of the Fas and TNFR recruits death domains, which activate caspase 8 triggering the caspase cascade activating caspase 3 leading to apoptosis. IAPs can be activated or activate transcription factors such as NFκB. IAPs block activation of caspase 2, 3, 7 and 9 preventing apoptosis. FasL – Fas ligand, FADD – Fas associated protein with death domain, TNF – tumour necrosis factor, TNFR – TNF receptor, TRADD, TNFR associated death domain, TRAF – TNFR associated factor, Cyto C – Cytochrome C, Apaf1 – apoptotic protease activating factor 1, AP1 – activator protein 1, NFκB – nuclear factor kappa light chain enhancer of activated B cells, NIK - NFκB inducing kinase, ASK1 –

apoptosis signalling regulating kinase 1, JNK-Phos – cJUN N terminal Kinase phosphorylation (Wei et al., 2008).

1.5.1 BIR domains

Baculovirus IAP repeat (BIR) domains are found in certain proteins when bound inactivate the protein allowing apoptosis to occur, however they also play a role in cell development. The BIR domain itself is structurally distinct, with a zinc-finger fold integrated into it (Hinds et al., 1999). It was shown that there were two types of BIR domains (Silke and Vaux, 2001; Verhagen et al., 2001). For inhibitors of apoptosis proteins, all forms of IAPs contain the type 1 BIR domain, however there were some from the family that also contained the second type of BIR domain, they are Survivin and Apollon (Takahashi et al., 1998; Verhagen et al., 2001).

The two types of BIR domains are distinguished by the presence or the absence of a deep peptide binding groove (Gyrd-Hansen and Meier, 2010). Type II BIR domains are more conserved then type I BIR domains (Cao et al., 2008). Type I do not contain this groove, whilst BIR type II, which generally target proteins that carry an IAP-binding motif (IBM) contain the groove (Cao et al., 2008; Gyrd-Hansen and Meier, 2010). IAPs may contain up to three BIR domains; however the function of each individual BIR domain is different allowing the IAP to contain specific properties and functions (Duckett et al., 1996).

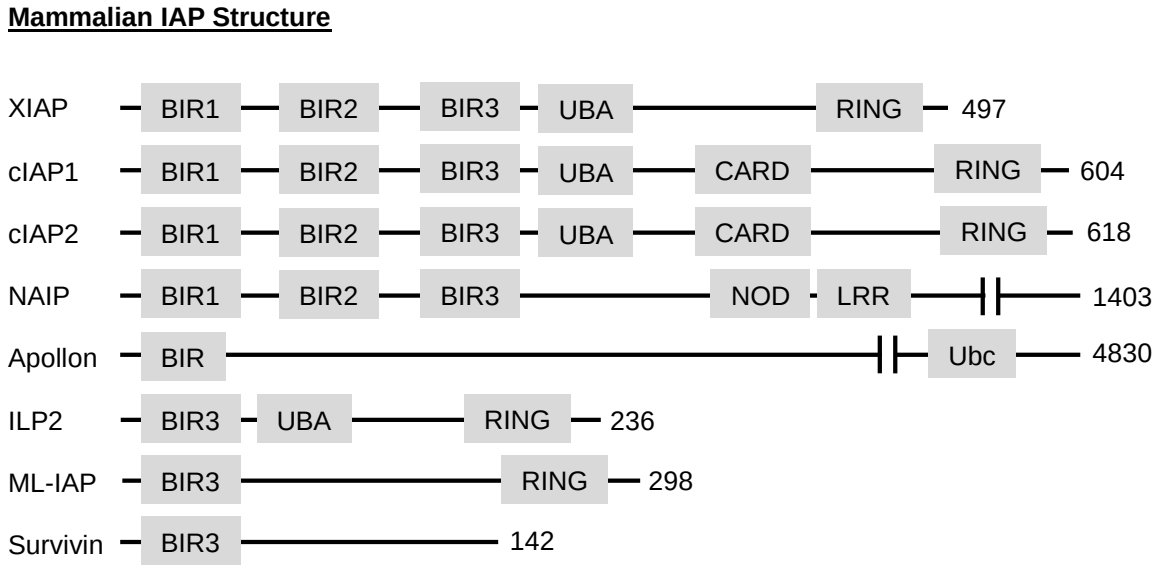


Figure 1.10: Molecular structure of the eight different mammalian IAPs

All IAPs have at least one baculovirus IAP repeat (BIR) domain, with some containing other features like a really interesting new gene (RING) finger or a caspase recruiting domain (CARD), ubiquitin associated domain (UBA), ubiquitin conjugase (Ubc), leucine rich repeat (LRR), nucleotide binding and oligomerisation domain (NOD); figures represent amino acid length (Beug et al., 2012; Wei et al., 2008).

1.5.2 XIAP

The XIAP protein structure is characterised by the containment of three BIR domains and a carboxy-terminal really interesting new gene (RING) finger domain (Cao et al., 2008). It has been documented that the BIR domain 1 of XIAP protein is capable of dimerisation, activating the NFκβ signalling pathway (Lu et al., 2007). The second and third BIR domains found in XIAP are responsible for interacting with the caspase proteins, specifically by blocking the active sites of caspase-3, -7, and -9 (Eckelman et al., 2006; Riedl et al., 2001). BIR3 specifically binds to capase-9 which is the initiator caspase (Shiozaki et al., 2003); the binding requires specific interactions between the surface groove found on BIR3 and the residue IAP Binding Motif (IBM) molecule found on the amino terminus of the small subunit of pro-caspase-9

(Shiozaki et al., 2003; Srinivasula et al., 2001), whilst BIR2 and the linker molecule between BIR1 and BIR2 binds to caspase-3 and caspase-7 (Huang et al., 2001; Riedl et al., 2001; Salvesen and Duckett, 2002). The linker molecule interacts with the substrate binding groove of the activated caspase molecule in a reverse orientation to the substrate, whilst the BIR2 domain interacts with the amino-terminus of the small subunit of the caspase molecule once exposed during its activation (Chai et al., 2001; Huang et al., 2001; Suzuki et al., 2001b; Varfolomeev et al., 2006).

However, XIAP is a more potent inhibitor of caspase-9 and caspase-3 then the other IAPs (Vince et al., 2007). Of all the IAPs, only XIAP directly inhibits caspase activity (Eckelman and Salvesen, 2006). Mutant mice lacking XIAP did not show phenotypic abnormality, and were not susceptible to apoptosis induced by different stimuli (Conze et al., 2005; Harlin et al., 2001; Vince et al., 2007). However, over-expression of XIAP has been shown to increase tumour cell survival (Hunter et al., 2007). XIAP is not only an inhibitor of apoptosis via inhibition of caspases, but can also promote pro-survival signalling, by activating the NFκβ and JNK pathways (Birkey Reffey et al., 2001; Hofer-Warbinek et al., 2000; Levkau et al., 2001; Lu et al., 2007; Sanna et al., 2002). It has also been shown in mice, that XIAP expression is gender specific, with a higher expression of XIAP in female mice compared to gender matched male mice (Siegel et al., 2011). In this study, to determine that the mRNA expression was not due to circulating oestrogen levels, a comparison of XIAP mRNA baseline levels were compared between ovariectomised mice and those replaced with 17β-oestradiol; baseline XIAP levels were significantly higher.

In some studies it has been shown that XIAP has a protective effect in a caspase independent manner. A study looking at expression of XIAP in human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAECs) on drug induced apoptosis, where caspase-3 activity was increased, showed that even though XIAP was able to inhibit apoptosis,

caspase-3 expression was not affected by XIAP (Kim et al., 2008). Therefore, it could be possible that XIAP inhibits apoptosis downstream of caspase 3.

VEGF is an important growth factor for the production of new vascular structures. Studies have shown that VEGF upregulates the production of XIAP in endothelial cells, suggesting its importance in VEGF-dependent cell survival (Kim et al., 2008). To understand the mechanism by which XIAP is implemented in cell survival, XIAP levels were monitored via Western blotting after treatment of HUVECs with different growth factors, VEGF, basic fibroblast growth factor (bFGF) and insulin like growth factor (IGF). Only VEGF caused an increase in XIAP levels (Kim et al., 2008).

XIAP is specific to certain cell types and is highly expressed in the skin, ovaries, uterus, prostate and testis in humans (Vischioni et al., 2006). Recently, Fuchs et al. (2013) have shown in an *in vivo* study that XIAP is expressed in the skin and hair follicle of mice and that with *XIAP* knockout, wound healing is compromised. In this study, it was demonstrated that with the knockdown of *Arts*, a direct antagonist to XIAP, wound repair was significantly enhanced with increased re-epithelialisation, and that bulge activity in the mouse hair follicle showed an increase in cell proliferation. To determine the exact effect of XIAP, a single knockout of XIAP also disrupted wound repair. To demonstrate the importance of Arts and XIAP in wound healing, a double knockout of both Arts and XIAP demonstrated that the enhanced re-epithelialisation seen in the Arts knockout mouse was abolished; indicating that XIAP plays an important role in wound healing.

1.5.3 cIAP1 and cIAP2

Two different cellular IAPs have been identified in mammals: cIAP1 and cIAP2. These IAP proteins are similar to each other in structure, function and localisation (Cao et al., 2008). Both types of cIAP proteins (1 and 2) are thought to have evolved from the XIAP protein since these three proteins contain three BIR domains that have similar subgroups, with the slight variation of having the CARD inserted between the BIR domains and the RING

finger; the RING finger domain has an additional function as an E3 ubiquitin ligase, which is capable of autoubiquitination, where the substrates of this activity are Smac/DIABLO, caspase-9 and RIP1 (Ma et al., 2006; Morizane et al., 2005).

cIAP1 was originally identified by its association with TNF-R2 via TRAF1 and TRAF2 (Rothe et al., 1995; Uren et al., 1996). cIAP1 is capable of binding to both caspase-7 and caspase-9, however is not able to directly inhibit their proteolytic activity (Eckelman and Salvesen, 2006; Tenev et al., 2005). Caspase-9 is inhibited by cIAP1 in the nucleus (Tanimoto et al., 2005). Studies have shown that cIAP1 can interact with the MAPK pathway by binding to the C-RAF protein kinase molecule (Dogan et al., 2008). cIAP1 contains both a BIR domain and a CARD domain, and in between these two structures is an ubiquitin-associated domain (Blankenship et al., 2009).

Studies with cIAP1$^{-/-}$ knockout mice show no phenotypic abnormality and appear to have no functional deficiencies in apoptosis initiated by a variety of stimuli (Conze et al., 2005; Harlin et al., 2001; Vince et al., 2007). Even though that would suggest that this specific IAP is not important for survival, increased levels of cIAP1 have been shown to increase tumour cell survival (Hunter et al., 2007). The main target for IAP antagonists is cIAP1. Low levels of cIAP1, lead to NFκβ signalling activation and induces TNFα production; this causes apoptosis in tumour cells by activating caspase-8 and affecting TNF-R1 death receptor signalling (Vince et al., 2007). TRAF2 recruits cIAP1 and cIAP2 to regulate the suppression of the noncanonical NFκβ pathway (Zarnegar et al., 2008). Reduced cellular levels of cIAP2 lowers the ubiquitination of RIP1, which converts the RIP1 function from a pro-survival molecule to a pro-apoptotic adapter protein (Bertrand et al., 2008). .

The pattern of expression for both cIAP proteins is generally the same in human tissues. It was shown that cIAP2 is more highly expressed in the basal layer of epithelial cells in the skin where proliferating cells reside (Vischioni et al., 2006). When looking specifically at cellular localisation cIAP2 was found in the cytoplasm, whilst cIAP1 was found also in the nucleus of many different

epithelial cells (Vischioni et al., 2006). Studies comparing the localisation of cIAP1, cIAP2 and XIAP show that cIAP1 is the most abundant IAP of the three in many human tissues including the prostate, testis, ovaries and uterus (Vischioni et al., 2006).

1.5.4 NAIP

Neuronal Apoptosis Inhibitory Protein (NAIP) was the first IAP member to be identified in mammals due to a deletion in infantile spinal muscular atrophy (Roy et al., 1995), and is regarded as a potent agent for cell survival, both *in vitro* and *in vivo* (Christie et al., 2007; Crocker et al., 2001; Davoodi et al., 2010a; Hutchison et al., 2001; Mikrogianakis et al., 2007). It is 160kDa in size and has three BIR domains found at the amino terminal region; each playing a slightly different role in respect to what it binds to. The second BIR domain, BIR2 functions to bind to, and inhibit the actions of caspase-3 and caspase-7 (Maier et al., 2002); whilst BIR3 binds to and inhibits caspase-9 (Davoodi et al., 2004). NAIP also contains an ATP/GTP binding site and a long carboxy terminal region with no known functional domain or motifs (Davoodi et al., 2010a; Davoodi et al., 2004).

Another structural feature that is present in the NAIP protein, which sets it aside from the other IAPs, is that it contains a nucleotide binding oligomerisation domain (NOD) and a leucine rich repeat (LRR). As with the different BIR domains having different roles, these two special features also play a role in the overall function of NAIP. It has been determined that NOD acts as an ATP/dATP binding site allowing for the interaction between NAIP and caspase-9 to occur, since unlike other IAP members this IAP needs ATP to bind to caspase-9 (Davoodi et al., 2004). NOD is also important in the activation of NFκB, The LRR is a regulator for the BIR domains by blocking their interactions with the caspases (Davoodi et al., 2010a).

It has only recently been investigated whether the full length of the NAIP protein or the specific BIR3 domain interacts with the caspase-9 apoptosome complex. However, in a recent study by Davoodi et al. (2010a) it was shown

that NAIP prevents apoptosis at the point of apoptosome formation and its inhibition cannot be stopped by IAP antagonists such as Smac/DIABLO. NAIP also appears to be involved in activation of pro-inflammatory caspase-1 (Fortier et al., 2005; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). NAIP can protect neurones from cell death by both caspase-dependent and caspase-independent pathways via its BIR3 domain (Mercer et al., 2000).

NAIP is regulated by the transcription factor Pax2, which directly binds to a specific recognition motif in the *Naip* gene promoter; Mutant mice heterozygous for Pax2 reduced the levels of NAIP mRNA expression by 50% (Dziarmaga et al., 2006). The level of NAIP expression is generally low in most tissues (Xu et al., 2002); NAIP has been shown to be active in neurones (motor, but not sensory) (Liston et al., 1996; Perrelet et al., 2000; Xu et al., 1997a; Xu et al., 1997b), the granulosa cells of the ovaries in mice (Matsumoto et al., 1999) and cell lines including Chinese hamster ovaries (CHO), Henrietta Lacks (HeLa) cervical cancer cell line and Rat-1 (Liston et al., 1996) and can be found in the human CNS, placenta, liver, spleen, lung and peripheral blood leukocytes (Maier et al., 2007).

1.5.5 Apollon

This cytosolic protein is one of the largest IAPs containing both the BIR domain in the amino terminal region and the ubiquitin-conjugating enzyme domain, motif at the carboxy terminal region; also at the C-terminal is a ubiquitin-carrier protein motif (Hauser et al., 1998). It is membrane-associated and contains only one BIR domain (Chen et al., 1999a; Hauser et al., 1998). Apollon can inhibit apoptosis induced by the IAP antagonist Smac/DIABLO, by promoting the ubiquitination and degradation of the mature form of the antagonist Smac/DIABLO (Hao et al., 2004), by specifically binding to the precursor of Smac/DIABLO (Qiu and Goldberg, 2005). Apollon can also bind to pro-caspase-9 preventing its cleavage (Hao et al., 2004; Qiu and Goldberg, 2005; Qiu et al., 2004), and the initiation of the caspase cascade (Srinivasula et al., 2001; Sung et al., 2007); However it does not inhibit the activity of caspase-3 (Qiu and Goldberg, 2005).

Expression of Apollon has been associated with neural progenitor cells indicating that Apollon is important in the proliferation of stem cells and neurogenesis (Sippel et al., 2009). Apollon plays a role in the control of cell death in early development; the knockout of *Apollon* results in defective development of the placenta; mouse embryos died *in utero* due to high levels of apoptosis in both the placenta and yolk sac (Ren et al., 2005). Mutations of Apollon leads to the up-regulation of p53, a tumour suppressor causing apoptosis in the mitochondria (Ren et al., 2005). High expression of Apollon has now been associated with malignancy (Sung et al., 2007). For example, Apollon is upregulated in brain tumour cell lines that are resistant to DNA damaging agents (Chen et al., 1999a).

1.5.6 ILP2

ILP2 (IAP like protein 2) is a homologue of XIAP. This protein is only expressed in testis in normal human tissue; and detected in lymphoblastoid (lymphocyte infected with Epstein-Bar virus) cells (Richter et al., 2001). ILP2 has no effect on apoptosis mediated via Fas and TNF; however, it inhibits apoptosis with high expression of Bax, or with the co-expression of caspase 9 with Apaf1.

1.5.7 ML-IAP

ML-IAP (Livin) is a 39kDa protein with a single BIR domain and a zinc binding RING domain, and like all other IAPs interacts with caspases. ML-IAP has a weak binding affinity with caspase-3, -7 and -9 (Vucic et al., 2005), however, it inhibits caspases indirectly by promoting the degradation of Smac/DIABLO via its E3 ubiquitin ligase activity (Kasof and Gomes, 2001; Lin et al., 2000; Ma et al., 2006). It also competes with the binding site on Smac/DIABLO, reducing XIAP-Smac/DIABLO binding, creating a pool of XIAP which can interact with the caspases to prevent apoptosis (Shin et al., 2005; Vucic et al., 2000). ML-IAP can be cleaved by caspase molecules; its truncated form has pro-apoptotic activity (Abd-Elrahman et al., 2009; Nachmias et al., 2003).

1.5.8 Survivin

One of the more studied IAPs is Survivin. It is the smallest IAP identified (Ryan et al., 2009), it has a single BIR domain in the amino-terminal region that is crucial for homo dimer formation, and also for protein-protein interactions with caspases (Chantalat et al., 2000; Verdecia et al., 2000); it was discovered that the BIR found in Survivin has a similar structure to the BIR2 of XIAP (Ambrosini et al., 1997; Uren et al., 1998) .

Survivin interacts in both the intrinsic and extrinsic pathways, counteracting different mediators including IL-3, FAS and caspases -3, -7 and -8 (Ambrosini et al., 1997; Azuhata et al., 2006; Mahotka et al., 1999; Mirza et al., 2002; Suzuki et al., 2000; Tamm et al., 1998; Zaffaroni et al., 2002).

Another hypothesis that has been suggested for the mechanism of Survivin, is that it is able to bind to Smac/DIABLO, displacing already bound IAPs allowing them to function by inhibiting apoptosis via the binding of caspases (Du et al., 2000; Verhagen et al., 2000). Survivin expression is high during foetal development, however is rarely seen in healthy adult tissue; up-regulation of Survivin is seen in the majority of cancer cells (Altieri, 2008; Li, 2003). Due to the high expression of Survivin in carcinoma cells, Survivin has become a protein of interest in developing new therapeutic targets for cancer treatment.

1.6 IAP Antagonists

1.6.1 Smac/DIABLO

The Second Mitochondria-derived Activator of Caspases (Smac)/Direct IAp Binding with LOw pI (DIABLO) protein (Du et al., 2000; Verhagen et al., 2000) is an endogenous antagonist of IAPs (Cossu et al., 2009). Even though this protein is generally found in the mitochondria, during the process of apoptosis it migrates into the cytosol (Verhagen and Vaux, 2002). It is capable of activating caspase-3, -7 and -9 by interfering with the IAPs binding to the caspases, mainly those that interact with caspase 9 (Du et al., 2000; Shiozaki and Shi, 2004; Verhagen et al., 2000; Wu et al., 2000). Smac/DIABLO as well

as other IAP antagonists have a conserved N-terminal IBM (Du et al., 2000). It is the amino terminal sequence that is important in the binding of Smac/DIABLO to the IAP molecules; mutations in this sequence can cause the protein to lose its function (Chai et al., 2000).

Smac/DIABLO is capable of interacting with cIAP1, cIAP2 and XIAP via its E3 activity, an ubiquitin ligase. In HeLa cells, Smac/DIABLO was shown to decrease the levels of both cIAP1 and cIAP2 by degradation; however it had the opposite effect on the levels of XIAP, with a slightly elevated level of expression (Yang and Du, 2004). Smac/DIABLO is capable of antagonising the effect of XIAP by binding to the same groove that is used to bind to the caspase molecules (Chai et al., 2000; Srinivasula et al., 2001). The degradating effects of Smac/DIABLO on certain IAPs (mainly cIAP1 and 2) is as a result of their auto-ubiquitination. Since Smac/DIABLO has an effect on cell survival, it has been of interest as a target protein. Recently, it has been identified that the Hip2 protein (a ubiquitin-conjugating enzyme, involved in cell cycle, and the suppression of cell death) interacts with Smac/DIABLO, and can also promote proteasomal degradation of mature Smac/DIABLO, preventing its interaction with IAPs (Bae et al., 2010).

1.6.2 Xaf1

XIAP associated factor (Xaf1) is one of the IAP antagonists that negatively regulates the effects of XIAP. Unlike the IAP antagonist Smac/DIABLO, which is a mitochondrial protein, Xaf1 is a nuclear protein which interacts with the XIAP-caspase complex, separating them (Liston et al., 2001). In particular, it is the caspase-3 interaction that is antagonised by the over expression of Xaf1 (Liston et al., 2001). The structure of this antagonist is that it does not contain an amino terminal tetrapeptide motif, suggesting that its interaction is specific, unlike the other IAP antagonists (Liu et al., 2000; Tu et al., 2009).

Whilst comparing Smac/DIABLO and Xaf1 with their interactions with XIAP, it was shown that Xaf1 binds preferentially to the BIR2 domain of XIAP, whilst Smac/DIABLO binds with higher affinity to the BIR3 domain (Liu et al., 2000).

Xaf1 has an additional role, which the other IAP antagonists do not share, in that it can also re-localise XIAP from the cytosol to the nucleus (Liston et al., 2001; Zou et al., 2006). Due to its association with XIAP, it is also considered to be a tumour suppressor; lack of this protein has been associated with many different cancer cell lines and human cancers (Byun et al., 2003; Fong et al., 2000; Lee et al., 2006; Ng et al., 2004).

The mRNA for Xaf1 is expressed in all normal adult and foetal tissue; whilst in different cancer cell lines, the expression is markedly reduced (Fong et al., 2000). Over-expression of Xaf1 in colon cancer enhances the activity of caspases, in particular caspase 8, via sensitisation of the cells to TNF Related Apoptosis Inducing Ligand (TRAIL)-induced-apoptosis (Leaman et al., 2002; Micali et al., 2007), an important member of the TNF family causing cell death to tumour cells only. Xaf1 over-expression also plays a role in the down regulation of IAPs such as XIAP, Survivin and cIAP2 through enhancement of TRAIL effects to trigger tumour regression (Tu et al., 2010). It's expression in melanoma cells is reduced compared to normal human epidermal melanocyte cells (Ng et al., 2004).

1.6.3 HtrA2/Omi

High temperature requirement A2 (HtrA2) is an IAP antagonist containing an IBM and a serine protease domain; both features are involved in the regulation of apoptosis (Hegde et al., 2002; Srinivasula et al., 2003; Suzuki et al., 2004; Verhagen et al., 2002; Yang et al., 2003). This protein, like Smac/DIABLO is located in the mitochondria, and is released into the cytosol during apoptosis (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001a; van Loo et al., 2002; Verhagen et al., 2002). The N-terminus of HtrA2 is cleaved with its release from the mitochondria. HtrA2 interacts with both BIR2 and 3 in XIAP, and can interact with cIAP1; it is not capable of interacting with Survivin (Suzuki et al., 2001a; van Loo et al., 2002; Verhagen et al., 2002). Overexpression of HtrA2 induces cell death in a caspase-independent manner, involving the shrinkage of cells without membrane blebbing and without the formation of apoptotic bodies (Suzuki et al., 2001a).

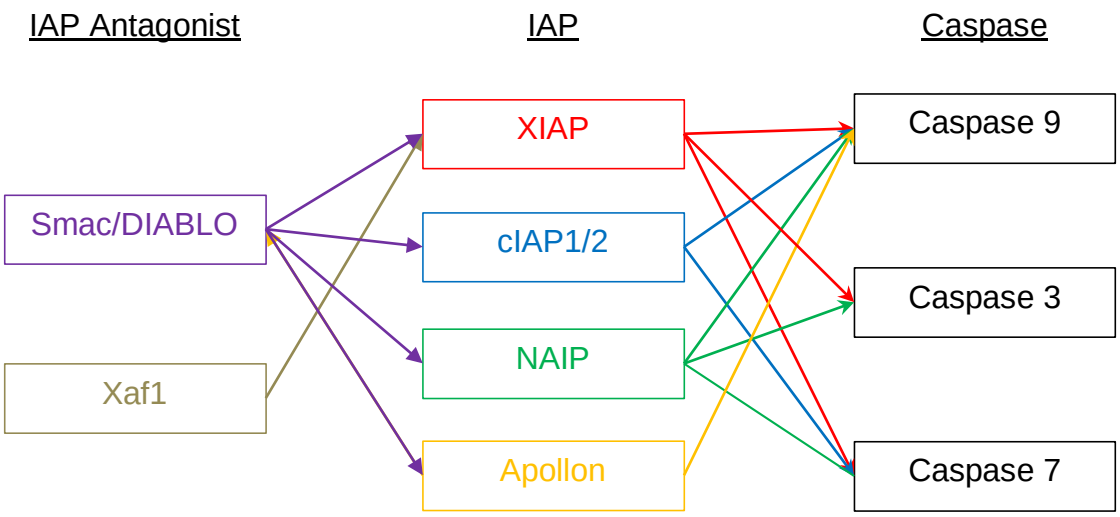


Figure 1.11: Basic overview of IAP and IAP interactions with caspases

General basic overview of the different interactions between inhibitors of apoptosis proteins (IAPs), IAP antagonists and initiator caspase 9 and effector caspases 3 and 7.

IAPs are regulated to mediate cell homeostasis and in pathology, for example cancer, this regulation is disrupted. IAPs therefore need to be tightly regulated to protect cells under stress conditions. They are also important in wound healing, especially in the remodelling phase where cells that are no longer required need to be removed from the site and to resume homeostasis; this function needs to be tightly controlled (Greenhalgh, 1998).

Oestrogen has been established to improve wound healing, and provide protection in skin (Stevenson and Thornton, 2007) topical 17β-oestradiol treatment has been shown to improve cutaneous wounds by accelerating the closure of a wound and wound collagen deposition (Ashcroft et al., 1999). In breast cancer cells, oestrogen has been demonstrated to regulate the levels of anti-apoptotic proteins such as Bcl2 (Frasor et al., 2003; Teixeira et al., 1995). Another study shows that in breast cancer, 17β-oestradiol activates the transcription factor NFκβ, a pro-survival mediator (Biswas et al., 2003; Frasor et al., 2009); in MCF7 cells, cIAP2 was a target gene to oestrogen receptor, qPCR demonstrated an upregulation of cIAP2 in the presence of

17β-oestradiol and TNFα, blocked by cells incubated with ICI 182, 780; NFκβ was shown to be highly up-regulated. In a study by Stanculescu et al. (2010), it was demonstrated that in breast cancer cells, 17β-oestradiol promotes cell survival through IAP activation. 17β-oestradiol was shown to increase levels of cIAP2 mRNA in combination with TNFα, but not cIAP1 and XIAP; also in the absence of cIAP2 via silencing the gene, the protective function of 17β-oestradiol was not observed. A different study by Bradford et al. (2010) demonstrated that in osteoblasts, oestrogen protect these cells by preventing apoptosis. This has been linked to its effect on caspase activity, in particular to inhibit effector caspases 3 and 7 (Bradford et al., 2010). This interaction could be a possible mechanism to protect cells when stressed; with the inhibition of effector caspases, apoptosis is prevented.

1.7 Oestrogen

Oestrogens are steroid hormones, commonly known as the primary sex hormones in females. In women, oestrogen can be found in three main forms circulating in the blood. A fourth form can be found circulating in the blood during pregnancy, which is produced by the liver of the human foetus; however, its role is still unclear (Coelingh Bennink et al., 2008; Hagen et al., 1965). The different types of oestrogens are: oestrone (E_1), 17β-oestradiol (E_2), oestriol (E_3) and oestetrol (E_4); they are all C_{18} steroids, with slight differences in the chemical composition and the number of hydroxyl (OH) groups found in each type (see Figure 1.12).

Each variation is important in its function since different levels of these hormones are found at different stages of the women's life (Watson et al., 2008). The most predominant oestrogen associated with the female reproductive phase is 17β-oestradiol. During and after menopause, oestone is the most significant oestrogen in the blood; whilst oestriol levels are higher in women during pregnancy (Watson et al., 2008). In men, it is mainly 17β-oestradiol that is converted from testosterone in peripheral tissue (Sherwin et al., 2011); levels vary depending on the levels of testosterone and the enzyme aromatase which converts testosterone into 17β-oestradiol.

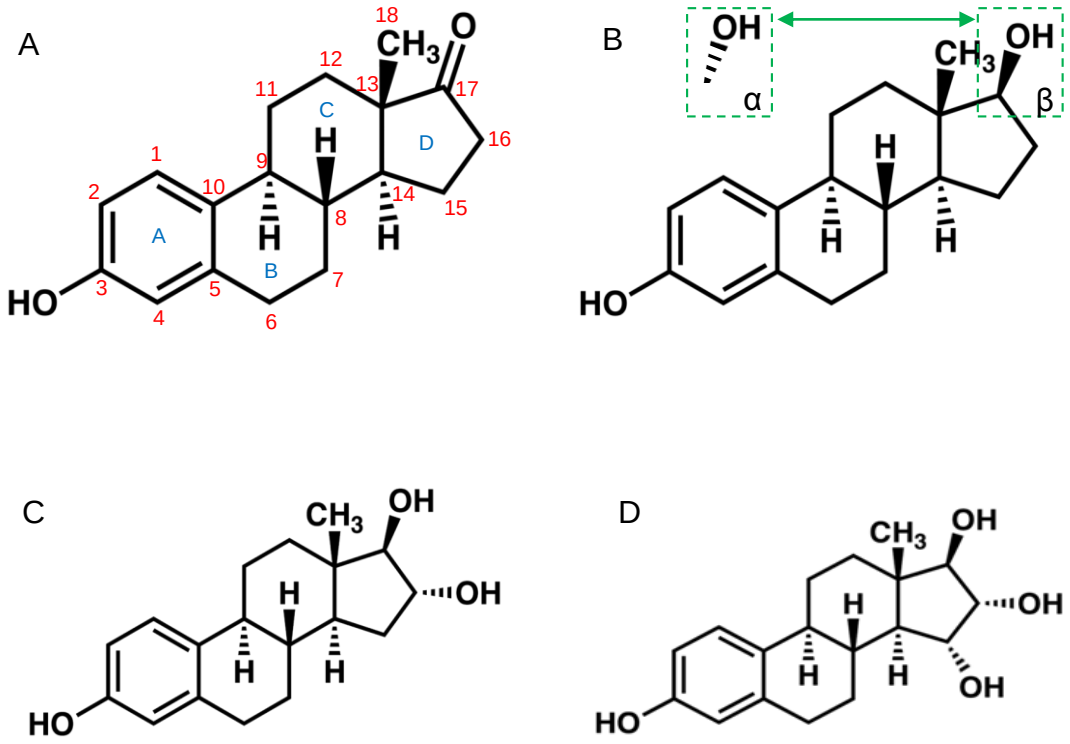


Figure 1.12: Structural composition of the different forms of oestrogen

C_{18} steroids, all containing one OH group attached to the benzene A ring at C_3. A) Oestrone, with a ketone (=O) attached to C_{17} on the D ring. B) 17β-*oestradiol, OH group at C_{17} on the D ring. Two isoforms exist, 17α-oestradiol the OH group sits below the plane of the D ring, altering its 3D structure and its affinity for the oestrogen receptors (green dashed box). C) Oestriol, has a second OH group attached to D ring at C_{16}. D) Oestetrol, has a third OH group attached to the D ring at C_{15}. (Coelingh Bennink et al., 2008)*

Oestrogen is able to act on cells via both the genomic and the non-genomic pathways (classical and non-classical). The genomic pathway involves intra-cellular receptors located in the nucleus or cytoplasm that act as transcription factors (Nilsson et al., 2001); whilst the non-genomic pathways involves a membrane bound receptor and secondary messenger signalling events (Silva et al., 2010). Circulating levels of 17β-oestradiol in men are 50-200pmol/L, while the levels in females vary depending on the stage of the menstrual cycle. During the follicular phase they are 70-500pmol/L, during the preovulatory

phase around 400-1500pmol/L, in the luteal phase 70-600pmol/L, and after menopause circulating levels are below 130pmol/L (Hornum et al., 1997).

1.7.1 Oestrogen biosynthesis

In females of reproductive age, the synthesis of oestrogen occurs principally in the ovaries, however other tissues (peripheral sources) such as the liver, skin, adipose tissue, adrenal glands and breasts also produce oestrogen, which are important sources after menopause (Simpson et al., 2000). Oestrogen is the terminal steroid in biosynthesis from cholesterol (see Figure 1.13).

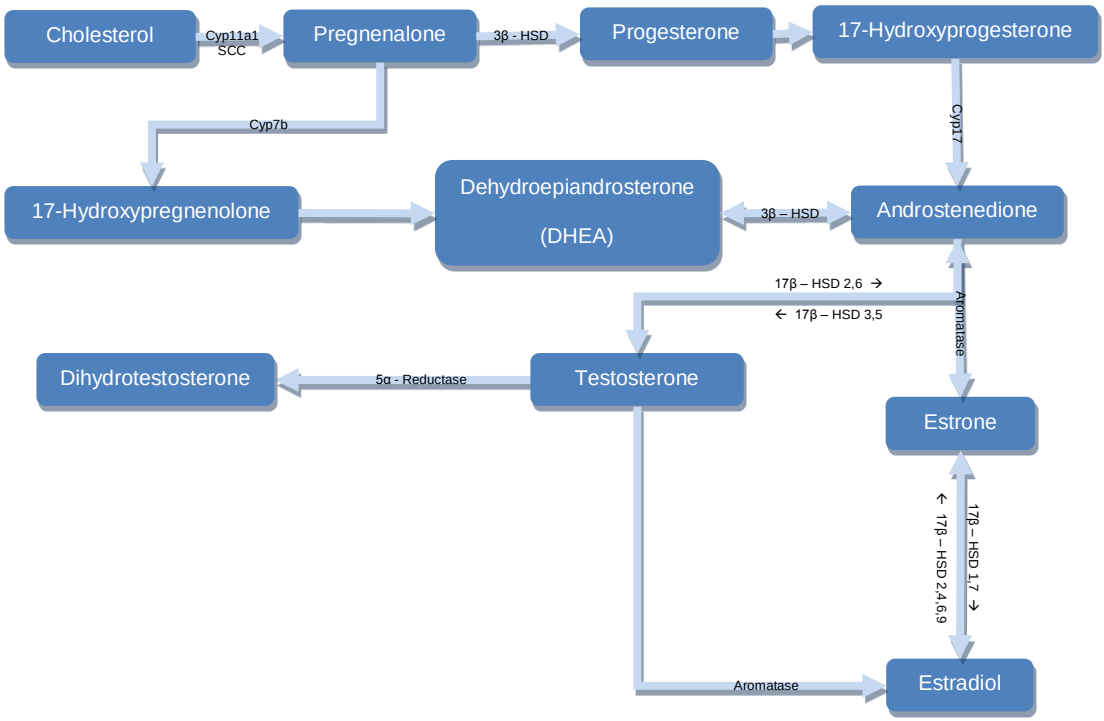


Figure 1.13: Flow chart representing the biosynthesis of oestradiol from cholesterol and the enzymes involved

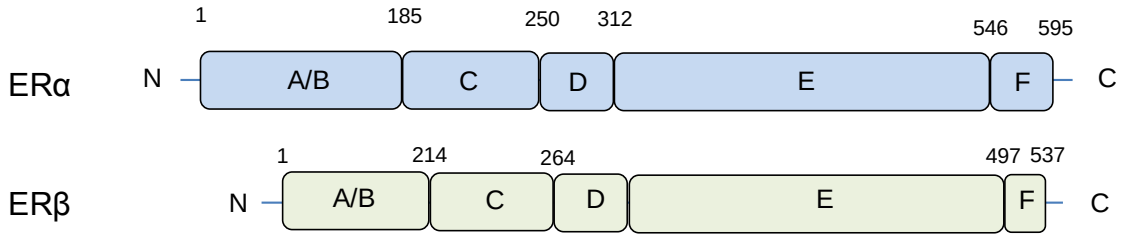
Oestrogen is produced from cholesterol in a number of stages involving different enzymes. DHEA and androstenedione are also secreted by the adrenal cortex in humans and some primates to act on peripheral cells (Martin et al., 2004; Rose et al., 1997). Cyp11a1 SCC – cholesterol side chain cleavage enzyme, HSD – hydroxysteroid dehydrogenase, Cyp7b – cytochrome P450

Aromatase is the final enzyme involved in the synthesis of oestrogen. Oestrogen can be synthesised in extragonadal sites, which allows it to act in a paracrine or intracrine manner (Simpson et al., 2000). The production of oestrogen in extragonadal tissue is dependent on circulating C_{19} precursors, DHEA and androstenedione, which are produced by the adrenal cortices of both sexes and from circulating testosterone in males. However, some tissues including the skin can synthesis oestrogen from cholesterol (Inoue et al., 2012).

1.7.2 Oestrogen action via the genomic pathway

The genomic pathway, also known as the classical pathway involves intracellular receptors that act as transcription factors. For many years, it was thought that there was only one oestrogen receptor that mediates all the pharmacological and physiological effects of oestrogen and their antagonists (reviewed by Nilsson et al. (2001) and Thornton et al. (2003a)). It was in 1996 when a second oestrogen receptor was identified in male tissue (Kuiper et al., 1996; Mosselman et al., 1996); they named the first ERα, and the second ERβ.

Oestrogen Receptors (ER) α and β belong to a nuclear receptor superfamily that include all the steroid receptors and are activated through ligand-receptor binding. They share a high degree of homology with regards to some of their functional domains, namely their DNA and ligand binding domains; however their N-terminal is more divergent (Hall et al., 2001). Their cell and tissue localisation and expression varies, indicating different functions in different cell types (Couse et al., 1997; Monje et al., 2001). Once a ligand such as 17β-oestradiol binds to an ER through a ligand-binding domain, it causes a conformational change in the receptor releasing the chaperone protein (heat shock protein), allowing interaction and binding to a specific DNA sequence known as the oestrogen response elements (ERE) and leading to an altered rate of transcription of oestrogen related genes (Nilsson et al., 2001). Ligand binding can either cause homo- or hetero-dimerisation of oestrogen receptors, depending on cellular expression.



Domain:	Homology:	Features:
A/B	18%	AF1
C	97%	DBD and dimerisation
D	30%	NLS
E	59%	NLS, LBD and dimerisation
F	18%	AF2

Figure 1.14: ERα and ERβ domains

Schematic representation of the oestrogen receptor α and β domains and their homology for each individual domain. AF – activation function, DBD – DNA binding domain, NLS – nuclear localisation site, LBD – ligand binding domain, N – amino terminal, C – carboxyl terminal (Garcia-Becerra et al., 2012).

Through the genomic pathway, there are two different mechanisms of action for oestrogen, ligand dependent and independent interaction. For the ligand dependent activation, in the absence of hormones, the receptors are in a state of seclusion by a multiprotein inhibitory complex found in the target cells (Hall et al., 2001). When the ligand binds to the receptor, it causes conformational changes, which allows it to bind to EREs upstream of the target genes (Hall et al., 2001; Walker et al., 1984). Tissue specific co-factor proteins allow the DNA-bound receptor to come into contact with the general transcription apparatus (Hall et al., 2001; McKenna et al., 1999). In ERα, there are two domains in which the transcriptional activity is mediated, one is found within the N-terminus (A/B domain) which is named activation factor 1 (AF-1), and AF-2 which is hormone dependant and is located in the ligand-binding domain (Tora et al., 1989). Like ERα, ERβ also contains AF2, but no AF1 domain is

found in human ERβ, however the AF2 domains in the two subtypes function differently (Hall et al., 2001). The transcriptional activity of the AFs are dependent on the recruitment of co-factors. Although these AFs function synergistically, they are also capable of functioning independently (Hall et al., 2001).

ER function can also be modulated by other factors in the absence of oestrogen; ERα is activated by epidermal growth factor (EGF) and insulin like growth factor 1 (IGF-1), increasing the expression of target genes (Smith, 1998). Oestrogen activated ER can also lead to the interaction of the receptor with a second DNA-binding transcription factor, generally preventing the binding of other transcription factors such as interleukin 6 (IL6), via its interaction with a subunit of NFκβ (Galien and Garcia, 1997; O'Lone et al., 2004; Stein and Yang, 1995). In breast cancer cells, cells expressing ERα show a reduced level of NFκβ activity in comparison with ER negative cells (Gionet et al., 2009; Nakshatri et al., 1997).

1.7.3 Oestrogen action via the non-genomic pathway

As opposed to the genomic pathway that is relatively slow (hours as opposed to minutes (Foradori et al., 2008)), the non-genomic pathway allows 17β-oestradiol to act rapidly by triggering a variety of secondary messenger signalling pathways (Silva et al., 2010) including the activation of adenylate cyclase and cAMP and the proto-oncogene Src causing the consequent activation of the Erk pathway, including both Erk1 and Erk2 in the Src/Ras/Erk cascade (Aronica et al., 1994; Kousteni et al., 2001; Migliaccio et al., 1996; Silva et al., 2010). This pathway is triggered and mediated by membrane associated or cytosolic ER (G-protein coupled receptor 30 (GPR30), now known as G-protein coupled ER 1 (GPER1) (Cheskis, 2004; Sharma and Prossnitz, 2011).

There are two ways by which the non-genomic pathway can influence gene expression. Phosphorylation of the nuclear ERα via Src/Erk and PI3K signalling can activate the receptor in the absence of oestrogen (Kato et al.,

1995; Silva et al., 2010; Stoica et al., 2000). Genes that are susceptible to oestrogen regulation do not always contain EREs. Interaction with another DNA binding transcription factor such as the activator protein 1 (AP1), binding to promoter sequence on target genes (Gaub et al., 1990).

ERs found on the membrane of the cell are G-protein linked and respond to oestrogen ligands (Carmeci et al., 1997; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997). Initially it was thought that these receptors are only found on the cell membrane, however, immunohistochemistry has demonstrated that this receptor could translocate and be found in the endoplasmic reticulum and nucleus (Evans et al., 2014; Revankar et al., 2007). They were identified in ER negative breast cancer cells, which still responded to 17β-oestradiol (Prossnitz et al., 2007).

Oestrogen has been shown to activate the MAPK signalling pathway. It has been demonstrated that the vascular protecting effects of oestrogen acting via ERα are mediated by non-genomic mechanisms through MAPK and Akt signalling pathways activating endothelial NO synthase (Chen et al., 1999b; Simoncini et al., 2000). In osteoblasts, oestrogen has a proliferative and anti-apoptotic effect through the MAPK signalling pathway (Kousteni et al., 2001).

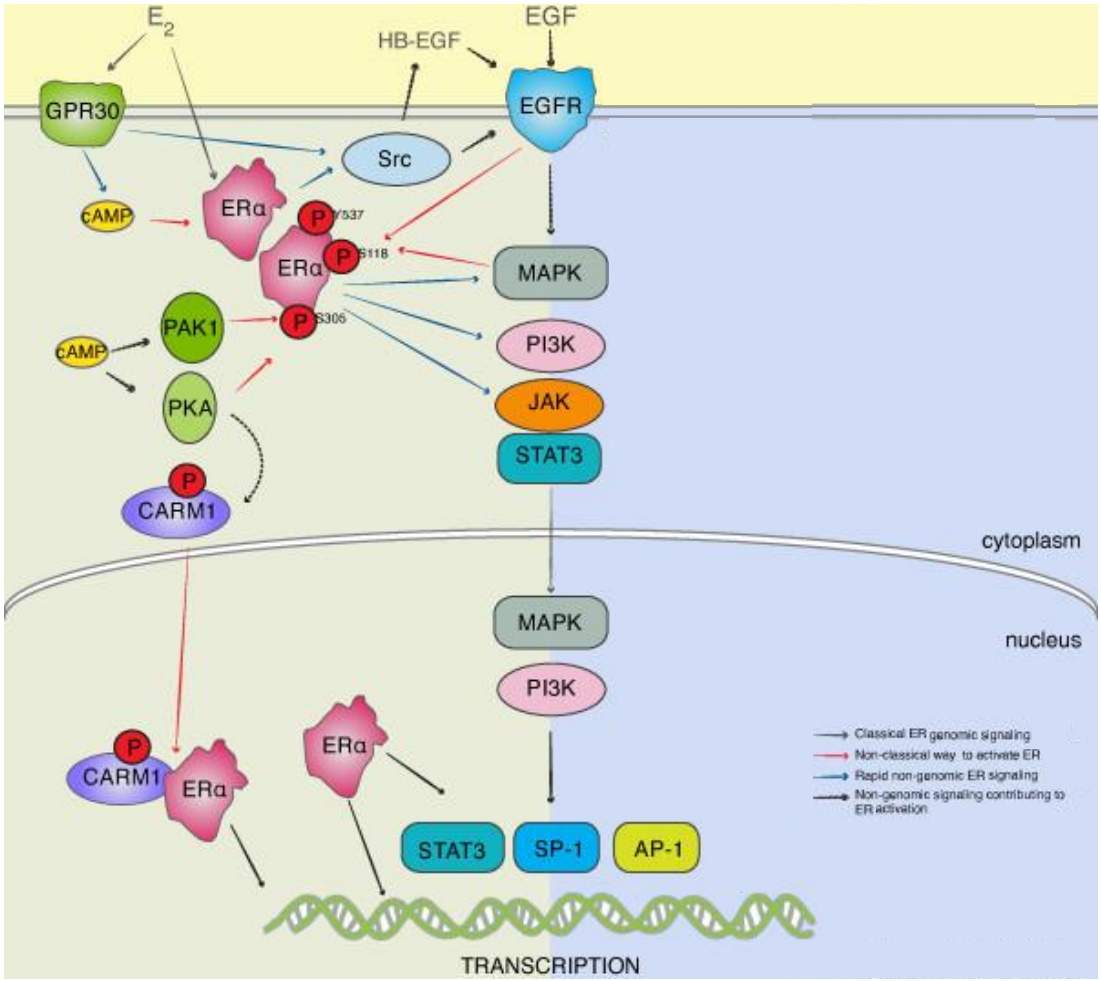


Figure 1.15: Oestrogen receptor genomic and non genomic pathway

Oestrogen receptors (ER) are capable of binding to DNA specific sequences either directly or indirectly via other transcription factors. ERs are capable of activating different signalling pathways including MAPK and JAK. cAMP is involved in activating oestrogen receptors induced by GPR30.

E2 - 17β-oestradiol, EGF – epidermal growth factor, PAK1 – Serine/threonine-protein kinase, PKA – cAMP dependant protein kinase, CARM1 – co-activator associated arginine methyltransferase 1, STAT3 – signal transducer and activator of transcription 3, SP1 – specificity protein 1, AP1 – activator protein 1. Adapted from Tanos et al. (2012)

1.7.4 The effect of oestrogens on wound healing

Studies have shown that ERs are expressed in different cells in human skin (Hasselquist et al., 1980) such as adipose cells (Pedersen et al., 1996), macrophages (Gulshan et al., 1990), fibroblasts (Thornton et al., 2003b), keratinocytes (Thornton et al., 2003b), melanocytes (Jee et al., 1994), and endothelial cells (Malet et al., 1991; Venkov et al., 1996). Many of these cells including fibroblasts, keratinocytes and immune cells are all important in wound healing, and therefore it would suggest that oestrogen has an effect on both normal skin homeostasis and during wound healing. Ashcroft et al. (1997a), investigating the role of 17β-oestradiol in wound healing in both humans and mice, showed that 17β-oestradiol substantially improved wound healing in aged humans, and in oestrogen depleted mice.

1.7.4.1 Effects of oestrogen on the inflammatory phase of wound healing

In both males and females in humans (Straub, 2007) and rodents (Kovats, 2012), oestrogen receptors are expressed in immune cells and their progenitors.

A histological study compared the inflammatory response and granulation tissue formation in response to oestradiol in rats ((Taubenhaus and Amromin, 1949) as reviewed by Calvin (2000)). Two groups of rats were compared, one virtually free of circulating hormones by the surgical removal of the male and female gonads and the adrenals, with a group which had intact adrenal glands and gonads, neither received any hormone therapy. The differences observed after wounding by subcutaneous implantation (to produce abscesses), were that in the animals that had their gonads and adrenals removed, the walls of the abscesses were thin with hardly any granulation tissue and fibroblastic response; while polymorphonuclear leucocytes were found in abundance, suggesting that the progression in healing from the initial inflammatory phase was lacking (Taubenhaus and Amromin, 1949) as reviewed by Calvin (2000).

More recent studies carried out by Ashcroft et al. (1999), demonstrated that application of topical oestrogen on wound sites of both elderly male and female patients significantly accelerates wound healing, with a reduced amount of neutrophil elastase and increased levels of fibronectin and collagen. In this study, elderly men (age 71.8 ±8.9 and 69.6 ±3.6 placebo and oestrogen) and women (age 72.5 ±7.1 and 76.3 ±5.6 placebo and oestrogen) were wounded (4mm punch biopsies in the left upper inner arm), and either topically treated with placebo or oestrogen for 24 hours. The wound area was significantly reduced in both sexes at similar rates with oestrogen treatment. There was also an increase in total collagen deposition after 7 and 80 days in patients treated with oestrogen. Markers for immune cells CD15 (neutrophils), and CD14 (monocytes) were used to compare the number of immune cells positive for ERα and ERβ 7 days after wounding in patients treated with either oestrogen and placebo. The results showed that there was a decrease in neutrophils and an increase in monocytes when treated with oestrogen for 24 hours. The chemotactic response of neutrophils was assessed using a 12 well chemotaxis chamber, and was significantly impaired in cells treated with oestrogen.

A later study by Campbell et al. (2010) compared the effect of ERα and ERβ specific agonists in ovariectomised mice in relation to inflammatory cells, and demonstrated that although signalling through ERα is sufficient to decrease inflammation, however alone, does not accelerate wound healing. With ovariectomised mice, treatment with DPN an ERβ antagonist at days -1, 0 and +1 of wounding; after 3 days of wounding, wound healing was accelerated. Indicating that ERβ has a negative effect on wound healing. Wound healing was delayed in mice that lacked oestrogen, either via ovariectomisation, or single knockout of ERα and ERβ. Interestingly, with ERβ knockout mice, treatment with oestrogen did not improve wound healing; however, it increased the number of neutrophils.

1.7.4.2 Effects of oestrogen on the proliferative phase of wound healing

Migration and proliferation of endothelial cells, fibroblasts and keratinocytes is essential in the proliferative phase of wound healing (Bello and Phillips, 2000; Park et al., 2011). Re-epithelialisation is one of the process involved in the proliferative phase of wound healing. Wound healing deteriorates with age, a study in male mice, showed that the rate of re-epithelialisation was delayed with age; with an increase in inflammatory cells contributing to the delay (Ashcroft et al., 1997b).

In rats, it has been shown that ERβ is responsible for activating tensile strength in wound healing *in vivo* and increased re-epithelialisation of the epidermis (tensile strength was calculated by measuring the maximal breaking strength at the wound edge by the wound area), whilst ERα was responsible for the increase in myofibroblast population (Novotny et al., 2011). To ensure that fibroblast differentiation into myofibroblasts were due to ERα agonists, human breast fibroblasts were treated with either ERα agonist PPT, or ERβ agonist DPN; after three days, there was an increase in myofibroblast population in cells treated with PPT, detected via immunohistochemistry, fibroblasts were stained with monoclonal antibody against αSMA to detect presence of myofibroblasts.

A recent study demonstrated that there is a difference in response to treatment to 17β-oestradiol in male and female gonadectomised mice (Gilliver et al., 2010). This study examined the effect of 17β-oestradiol on re-epithelialisation. Castrated males healed faster than ovariectomised female mice, however, when males and female mice were administered with oestradiol, wound healing was impaired in males and enhanced in females. This indicates that while 17β-oestradiol is beneficial in females, it can have a negative impact on males. Its inhibition was concluded to be via ERα; since in castrated ERβ null mice, there was a delay in wound healing and re-epithelialisation, whilst this was not seen in castrated ERα null mice (Gilliver et al., 2010).

Aging healthy females show a reduced rate of cutaneous wound healing, however their quality of scarring is improved, which would suggest that while oestrogen is required for wound healing, it has a negative impact on scarring (Ashcroft et al., 1997a). In this study, it was also observed that levels of TGFβ1 were reduced, and with an increase in TGFβ1 secretion by fibroblasts induced by 17β-oestradiol, wound healing was improved. In an *in vitro* study, human dermal fibroblasts unwounded monolayer treated with 17β-oestradiol caused an increase in the secretion of TGFβ1, however, in mechanically wounded cells, secretion of TGFβ1 was reduced (Stevenson et al., 2008a). This indicates that fibroblasts are target cells for 17β-oestradiol, but that the pathways involved are complex and that 17β-oestradiol could have different roles in wound healing responses.

Interestingly, activation of different ERs has different effects on TGFβ1 secretion, ERα antagonists in human keratinocyte cell line (NCTC2544) stimulated cell proliferation and increased TGFβ1 secretion, however, ERβ agonists, increased cell proliferation but did not increase secretion of TGFβ1 (Merlo et al., 2009). TGFβ1 was involved in keratinocyte cell migration in a scratch wound assay, with TGFβ1 treatment, keratinocyte cell migration was increased; this increased migration was also seen when cells were treated with 17β-oestradiol. With TGFβ1 antagonists, migration was inhibited, this was also seen with ERα agonist, but not with ERβ agonist (Merlo et al., 2009) indicating that ERβ is important in keratinocyte migration.

1.7.4.3 Effects of oestrogen on matrix formation and remodelling

Oestrogen has been shown to modulate the remodelling phase of wound healing. Delays in age related wound healing are thought to be due to low levels of TGF-β1 in humans (Ashcroft et al., 1997a; Neurohr et al., 2006), a decrease in collagen synthesis in humans (Ashcroft and Mills, 2002), and an increase in the enzyme elastase in humans (Herrick et al., 1997; Marini et al., 2010). Depending on the dose of oestrogen and when it is applied, it may have different effects on collagen deposition (Marini et al., 2010; Ramamurthy et al., 1999). During the remodelling phase of wound healing, collagen

synthesis detected by hydroxyproline assay, increased with oestrogen treatment in both male and female patients (Ashcroft et al., 1999).

Pirila et al. (2001) showed that in rats, the administration of 17β-oestradiol did not affect type I collagen deposition in Sham rats when wounded, however, it increased collagen deposition to Sham levels in ovariectomised rats. Ashcroft et al. (1997b) demonstrated via immunostaining that collagen deposition was reduced with age in male mice, and that when wounded, collagen organisation was comparable to normal dermis. A study by Markiewicz et al. (2013) compared collagen mRNA expression, and total collagen from ERα and ERβ knockout female mice. Dermal fibroblasts were cultured and collagen mRNA expression determined via qRT-PCR and total collagen by hydroxyproline assay. It was observed *in vivo* that ERα knockout mouse wounds showed a significant increase in collagen production compared to wild type, with a significant decrease in collagen with ERβ knockout mice. However, *in vitro*, this was not detected with dermal fibroblast culture. Both ERα and ERβ knockout mice showed an increase in collagen mRNA expression compared to wild type. Treatment of dermal fibroblasts with 17β-oestradiol increased collagen mRNA expression in wild type, ERα and ERβ knockout mice, which could indicate that 17β-oestradiol affects collagen production through another pathway such as through GPR30; to confirm ERα and ERβ conjunctive knockout should be established and dermal fibroblasts treated with 17β-oestradiol.

1.7.4.4 Effects of oestrogen on angiogenesis

Both ERα and ERβ are expressed in blood vessels (both endothelial and smooth muscle cells) (Karas et al., 1994; Losordo et al., 1994; Venkov et al., 1996). Oestrogens are capable of inducing rapid vasodilation, and modulate proliferation via down regulating thrombospondin 1 a negative regulator of angiogenesis (Sengupta et al., 2004). And can also regulate proliferation via apoptosis as a protective effect when HUVEC cells are treated with TNFα (Spyridopoulos et al., 1997). Oestrogens increased the synthesis of VEGF in mechanically wounded dermal papilla cells cultured from female scalp hair

follicles; however decreased levels in mechanically wounded dermal sheath cells and had no effect in mechanically wounded dermal fibroblasts (Stevenson et al., 2008b). VEGF is an important factor for angiogenesis and for the stimulation of re-epithelisation in wound healing (Stevenson et al., 2008b). This could indicate that the importance of dermal fibroblasts in influencing angiogenesis is not affected by oestrogens.

1.8 Aims

Although improved wound healing in anagen hair bearing skin has been attributed to the presence of hair follicle cells, human dermal fibroblasts derived from different anatomical regions, scalp, abdomen and breast display differences in cell migration and proliferation. However, a study looking at similar anatomical regions bearing terminal and vellus hair follicles have not been conducted.

It is also well established that 17β-oestradiol plays an important role in wound healing and has direct effects on both human dermal fibroblasts and hair follicle fibroblasts in culture.

More recently, the family of IAPs, in particular XIAP have been shown to have a significant role in wound repair in a murine model. Furthermore, there appear to be gender differences in the regulation of XIAP.

Therefore, this study will compare the expression of different IAPs and IAP antagonists *in vivo* in mice and *in vitro* in human dermal fibroblasts. The expression of IAPs will be examined in mouse skin following depilation, as plucking induces a wound healing response to the hair follicle and orchestrates the hair follicles into re-entering anagen of the hair cycle.

In order to establish whether there are differences in the expression for IAPs and their antagonists in human skin, scalp and facial skin biopsies derived from the same female donors will be used to culture dermal fibroblasts. Immunofluorescent staining will be carried out to establish expression and localisation of IAPs and their antagonists in different wounding environments.

To explore further the differences in the dermal fibroblasts derived from human hairy (scalp) and non-hairy (facial) skin in terms of wound healing, comparisons in migration, viability and proliferation will be conducted. Since XIAP has been associated with wound healing, mRNA expression of IAPs and their antagonists will be carried out under wounding and non-wounding conditions.

Fibroblasts are known to respond to 17β-oestradiol, and studies have shown expression of oestrogen receptors, however, a comparison of oestrogen receptors between dermal fibroblasts derived from terminal and vellus hair bearing skin has never been established.

The responses of the dermal fibroblasts to 17β-oestradiol in terms of migration, proliferation and IAP and their antagonists' expression will be conducted to compare between dermal fibroblasts derived from terminal and vellus hair bearing skin.

A major pathway in which XIAP functions, is through the caspase cascade, therefore, caspase activity will be monitored in dermal fibroblasts from terminal and vellus hair bearing skin under wounding and non-wounding conditions, in the presence of 17β-oestradiol as a cell protector and with embelin, to remove XIAP activity.

Not only will this highlight important differences between dermal fibroblast populations from the same person, but will also help to identify the relationship between oestrogen and IAPs in human skin, which to date has not been established.

Chapter 2

EXPRESSION OF IAPS IN MOUSE AND HUMAN SKIN

2 Expression of IAPs in Mouse and Human Skin

2.1 Introduction

Hair follicles are a good model system to study tissue remodelling in an adult, as their lower bulb region is regenerative under normal and different physiological conditions (Ito et al., 2007). By inducing anagen (the growth phase), the process of tissue remodelling begins (Paus and Foitzik, 2004). Cell proliferation and differentiation during anagen, and apoptosis during catagen are tightly controlled.

2.1.1 Mouse and human models

Mice and other rodents are often used for studies of hair growth, as well as models for wound healing, tissue remodelling and regeneration. It has been demonstrated that mice provide good models to study the hair cycle, since synchronised hair follicle cycling can easily be obtained via depilation (Stenn et al., 1996; Stenn and Paus, 2001). However, inducing anagen via depilation also causes a wound healing response around the hair follicle (Hsu et al., 2008; Matsuo et al., 2003). Inflammatory cells such as T- and B-lymphocytes are recruited to the outer root sheath of the hair follicle, and perifollicular mesenchyme following depilation to induce anagen (Paus et al., 1998). The hair follicle is forcibly removed from its dermal sheath; apoptosis is activated to remove injured cells, which also induces the hair follicles into the anagen or the growing phase (Matsuo et al., 2003). The spontaneous hair cycle is less synchronised postnatally in mice; the first anagen wave appears approximately 12 days after birth, while the second cycle begins approximately 28 days after birth (Muller-Rover et al., 2001). With age, the hair cycle becomes less synchronised, therefore, to study the different stages of the hair follicle cycle, anagen is generally induced via depilation (Paus et al., 1990). However, in humans all hair follicles cycle in an asynchronous manner, therefore it is more difficult to study the hair cycle in humans.

2.1.2 Expression of IAPs and their antagonists in skin

In the murine hair follicle, the two most studied members of the IAP family are Survivin (Grossman et al., 2001) and XIAP (Fessing et al., 2006; Fuchs et al., 2013). Survivin expression in the skin varies depending on the species, in rats, a study revealed that Survivin was expressed in the epidermis (Iskandar and Al-Joudi, 2006), however, in adult murine skin, the expression of Survivin in the epidermis was not found (Grossman et al., 2001). XIAP mRNA expression was analysed in wild type and *downless* mice (mice with Edar knockout, a member of the TNFR superfamily which is highly expressed in the mouse hair follicle during the anagen-catagen transition (Fessing et al., 2006)). XIAP expression was decreased by 5 fold in *downless* mice compared to wild type. Using immunofluorescent staining XIAP expression was decreased in the outer root sheath and the hair matrix in catagen hair follicles in *downless* mice compared to wild type, however it still remained strongly expressed in the dermal papilla (Fessing et al., 2006). More recently the expression of XIAP, using immunfluorescent staining, has been demonstrated in the bulge (stem cell region) of telogen hair follicles in the mouse (Fuchs et al., 2013).

Survivin expression has also been identified in human skin and hair follicles (Botchkareva et al., 2007); it was located in the cytoplasm of keratinocytes in the basal layer of the epidermis, while in the hair follicle, it was located in the hair matrix and outer root sheath. This study also investigated the functional importance of Survivin in keratinocyte cell proliferation. Using immunofluorescence the hair follicle was stained with a proliferative marker Ki-67 and Survivin antibody. Cells in the outer root sheath expressing Ki-67 also expressed Survivin, suggesting that Survivin may contribute to the ability of cells to proliferate. Also with immunohistochemical analysis of catagen hair follicles, this study demonstrated that the expression of Survivin in the hair matrix decreased during catagen, presumably to allow apoptosis to occur for follicle regression.

A study by Vischioni et al. (2006) compared the expression of XIAP, cIAP1 and cIAP2 in different human tissues via immunohistochemistry, including the skin and hair follicle. This study demonstrated that XIAP, cIAP1 and cIAP2 were all present in human epidermis and dermis, including nerves, sebaceous glands, sweat glands and the hair follicle; however, the specific compartments of the hair follicle that expressed these proteins were not individually assessed. Furthermore, this study provided no data with regards to whether the skin was obtained from male or female donors, nor the specific anatomical location from which it was derived.

In the human hair follicle cycle, IAPs are important in regulating catagen, the regression phase (Botchkareva et al., 2006). Apoptosis in the hair follicle signifies catagen, where the hair matrix undergoes apoptosis. The anagen-catagen transition can be induced by pro-inflammatory cytokines such as TNFα (Hoffmann et al., 1996); and inhibitors of apoptosis proteins are reduced (Botchkareva et al., 2006). During catagen the hair follicle outer root sheath also shows high expression of caspase 8 and caspase 3 activation (Peter and Krammer, 2003).

To date, the expression of other members of the IAP family have not been identified in murine skin and the hair cycle. However, a recent study by Fuchs et al. (2013) has demonstrated that in murine skin, the expression of XAIP was required for wound healing. While *XIAP* knockout mice were viable and displayed no phenotypical differences, loss of XIAP abolished re-epithelialisation, and wound repair was markedly delayed compared to wild type.

2.1.3 Importance of the hair follicle in wound healing

Hair follicles also contribute to wound healing in human skin, it has already been established that hairy skin heals better than non-hairy skin (Jahoda and Reynolds, 2001; Martinot et al., 1994); and that hair follicle cycling plays a major role in improving wound healing (Ito et al., 2005; Levy et al., 2007). In a murine model, improved wound healing has been related to re-

epithelialisation rather than wound closure, where the presence of anagen hair follicles are important for re-epithelialisation (Ansell et al., 2011).

In mice, the body is covered in terminal hair follicles, whilst in humans, the majority of the hair follicles are vellus, with terminal hair follicles found mainly in the scalp. The human hair follicle also differs considerably from the murine hair follicle, in that in certain regions e.g. scalp, beard and axilla, it is androgen dependant (Randall et al., 2000). In the human scalp approximately 90% of the hair follicles are in the anagen phase of the hair cycle, and the ratio of terminal to vellus hair follicles is 9:1 (Whiting, 1996). However, it is not possible to know whether a particular hair follicle is in early or late anagen, which could last for up to 7 years in the human scalp. Their asynchronous nature also makes it difficult to study the effects of hair cycling in the context of wound healing.

A major difference when comparing human skin from different regions is the type of hair follicle present, which can be either vellus or terminal hair follicles. However, to date, there have been no studies comparing the expression of different IAPs in human skin containing predominantly vellus or terminal hair follicles.

2.1.4 Aim

Since changes in the expression of Survivin in the murine hair cycle has already been established, the expression of cIAP1, cIAP2, XIAP and Apollon, (NAIP was not expressed in mouse hair follicles at any stage of the hair cycle via qRT-PCR, unpublished data), along with the IAP antagonists DIABLO and Xaf1 will be determined in murine skin at day 3, 5, 12, 19 and 21 after depilation. Due to the length of the murine hair cycle, this will include anagen, catagen and telogen.

The first aim of this chapter was to establish whether the immunohistochemical expression of IAPs and IAP antagonists in haired mouse skin, is altered following depilation, which not only induces a wound healing response to the

hair follicle, but initiates the anagen phase and the start of the remodelling phase of the hair follicle in a new hair cycle to produce a new hair fibre.

The second aim of this chapter was to compare the IAP expression of NAIP, cIAP2, Apollon and XIAP and their antagonists DIABLO and Xaf1 in human skin containing predominantly terminal hair follicles or vellus hair follicles in skin derived from the same donor. ML-IAP and ILP2 are generally associated with cancer, and therefore will not be studied. Hair follicles in the scalp are predominantly in anagen and can last between 5-7 years. These hair follicles are not synchronised, some could be in early anagen, whilst others are in late anagen; therefore, at different stages of the hair cycle the expression of IAPs and their antagonists are difficult to ascertain. Since human skin cannot be depilated to synchronise the hair cycle, the SACPIC staining method was used to distinguish whether hair follicles were in the anagen, catagen, or telogen phase of the hair cycle.

Human scalp containing terminal hair follicles, where ~90% of the hair follicles are in the anagen phase, was compared with corresponding facial skin of the same donor which contain small, fine and unpigmented vellus hair follicles. A comparison of skin from the same donor is ideal to determine whether the expression of IAPs in human skin differ between vellus and terminal hair bearing skin.

2.2 Material and Methods

2.2.1 Tissue samples

2.2.1.1 Mouse tissue

C3H male mice were used for experimental purposes. To determine the effect of depilation on IAP levels, sections obtained from mice at specific time points, were collected according to previously published procedures (Paus et al., 1999) obtained under the project licence available via the Centre for Skin Sciences, carried out by Dr Andrei Mardaryev trained and authorised to work with animals. Depilation was performed by the brief application of hot wax/rosin mixture to the dorsal skin of mice that were 7 weeks old, and in the full anagen stage of the hair cycle. The hair shafts were plucked using the wax/resin mixture to initiate a homogenous anagen wave across the entire depilated area; depilation induces an early wounding response and the entry of the hair follicle into early anagen. The time points in which the skin sections were obtained were 3-5 days after depilation, 12 days after depilation, 19 days after depilation and 21 days after depilation. The skin samples were mounted in OCT media and stored at -80˚C, (see Figure 2.1).

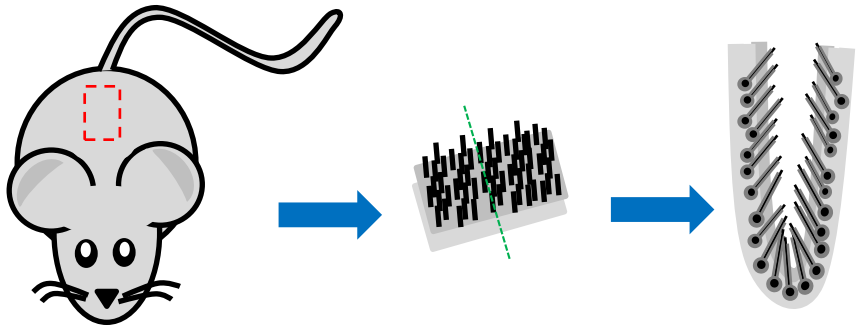


Figure 2.1: Mouse skin isolation

7 week old C3H mice were raised and a patch on the dorsal skin was depilated (area highlighted in dashed red box) and at different days after depilation, DP3, 5, 12, 19, and 21, mice were sacrificed to obtain the depilated skin section. Cut skin samples were folded (marked green dash line) and placed in OCT for cryosectioning.

2.2.1.2 Human tissue

Skin samples were obtained from healthy donors with full consent and ethical approval after undergoing plastic surgery. The skin samples were taken from female subjects (n=3, age range 50-57 - Table 2.1) following facelift surgery. The tissue was placed in transporting medium Dulbecco's Modified Eagle Medium (DMEM) (Sigma), supplemented with 10% foetal bovine serum (FBS) (GIBCO), 500Units/mL penicillin and 500µg/mL streptomycin (Pen/Strep) (GIBCO), 0.75µg/mL fungizone (GIBCO), glutamax (GIBCO)) and stored at 4˚C for up to 12 hours. Skin from each donor was divided into the terminal hair bearing scalp region and the non-hairy skin containing vellus hairs of the facial skin (Figure 2.2). This allowed a direct comparison of hairy and non-hairy skin from the same donor. Tissues for cryosectioning were frozen within 24 hours in OCT at -80˚C.

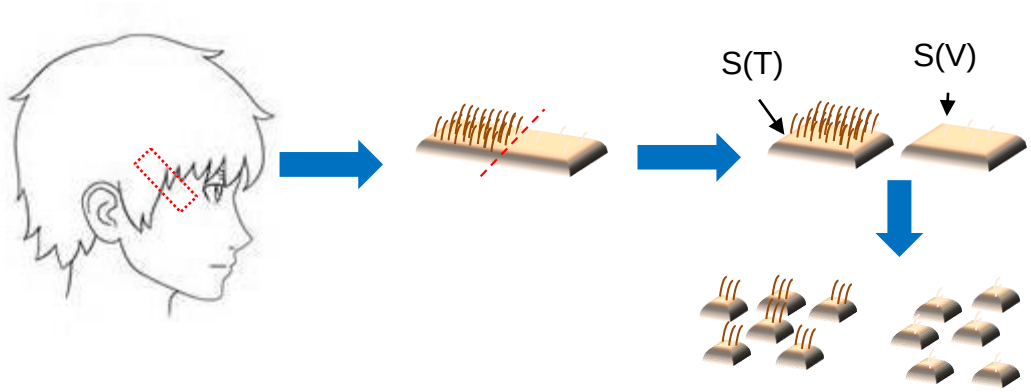


Figure 2.2: Terminal and vellus hair bearing skin preparation

Skin samples from female face lift patients – marked red dashed box were isolated from terminal hair bearing [S(T)] and vellus hear bearing skin [S(V)] – dashed red line. Skin samples from both terminal and vellus hair bearing skin were then cut into smaller 1cm^2 pieces frozen in OCT for cryosectioning.

Donor ID	Gender	Age	S(T)	S(V)
OK18	Female	56	✓	✓
OK26	Female	50	✓	✓
OK27	Female	57	✓	✓

Table 2.1: Donor sample for cryosectioning for IAP and IAP antagonist immunofluorescent and SACPIC staining

Three donor samples were used for cryosectioning to analyse IAP and IAP antagonist localisation and expression and SACPIC staining. S(T) – terminal hair bearing skin, S(V) – vellus hair bearing skin.

2.2.2 Cryosectioning

Frozen mouse tissue samples (~1cm x 2cm folded) were secured onto a cryostat chuck using OCT media and frozen using QuickFreeze solution/ spray. The cryostat temperature was set to -20˚C. The tissue sample was oriented in the cryostat in order to obtain 8µm longitudinal sections of the tissue where the entire hair follicle structure could be seen as well as the epidermis. The tissue sample was maintained at -24˚C during sectioning. Correct orientation was checked at the start of the sectioning by staining with haematoxylin for 30 seconds and the sections observed through a light microscope for definitive features. All sections were captured on charged slides (Poly-L-Lysine coated – Sigma; diluted 1:10 in deionised water) for electrostatic coupling, which allows for a more secure attachment of the section to the slide to withstand the staining procedure. All sections were stored at -80˚C for further use.

The human skin was cut to pieces of approximately $1cm^2$, orientated on the cryostat and longitudinal sections (of the hair follicle where available) were cut at 7µm. Sections were cut at -24˚C and stored at -80˚C on Poly-L-Lysine coated slides for future use.

2.2.3 SACPIC staining of human skin

All the solutions required for SACPIC staining were prepared before the experimental procedure, fresh solutions of ethanol and picric acid were made on the day of staining.

Frozen sections on slides were thawed and fixed by immersing in ice cold acetone for 15 minutes. Sections were rinsed twice in water gently and left to dry at room temperature. Slides were immersed in Celestine blue (Sigma-Aldrich) stain for 5 minutes then rinsed in tap water. They were then stained in Gill's haematoxylin (Sigma-Aldrich) for 5 minutes and rinsed in tap water. To stain the nucleus blue, the sections were placed in Scott's tap water for 2 minutes before rinsing in normal tap water. Sections were immersed in Safranin (Sigma-Aldrich) for 5 minutes before rinsing in tap water. Once the solutions had been washed from the slides, the sections were immersed in clean tap water for 1 minute, before placing in 70% ethanol, then 95% ethanol consecutively for 1 minute each. The slides were immediately incubated in a solution of absolute picric acid (Sigma-Aldrich) /Ethanol (ratio 1:1) for 3 minutes. Without washing, the slides were placed directly in 95%, then 70% ethanol for 1 minute, before finally hydrating with tap water for a further minute. The sections were stained with picro-indigo carmine (Sigma-Aldrich) for 2 minutes before rinsing in tap water, and then dehydrated by incubating in 70%, then 95%, then absolute alcohol for 5 minutes at each concentration. Sections were cleared in a solution of histoclear (National Diagnostics) /ethanol (ratio 1:1) for 4 minutes, before immersing in 100% histoclear for an additional 4 minutes. Slides were mounted using Histomount onto a coverslip and observed under a light microscope.

Reagent:	Staining Purpose:
Celestine blue	Connective tissue and outer root sheath
Gill's haematoxylin	Nuclear staining
Scott's tap water	Clear nuclear staining
Safranin	Non keratinised inner root sheath
Picric acid/ethanol	Keratinised hair fibre and inner root sheath
Picro-indigo carmine	Cells in mitosis, bulge and dermal papilla

Table 2.2: SACPIC staining reagents

The purpose of the individual staining reagents for SACPIC staining in the skin and hair follicle.

2.2.4 Immunofluorescence

Antibody (type):	Raised in:	Manufacturer:	Analysed in:	Dilution Ratio:
NAIP (1˚)	R	SantaCruz (SC11062)	H, M	1:50
cIAP1 (1˚)	R	Abcam (Ab108361)	M	1:100
cIAP2 (1˚)	R	Abcam (Ab32059)	H, M	1:1000
XIAP (1˚)	R	Abcam (Ab21278)	H, M	1:100
Apollon (1˚)	R	Abcam (Ab19609)	H, M	1:100
Xaf1 (1˚)	R	Imgenex (Img379)	H, M	1:100
DIABLO (1˚)	R	Imgenex (Img5753)	H, M	1:300
FITC (2˚)	D anti R	Abcam (Ab98502)	H, M	1:200

Table 2.3: Antibody dilution ratio used for immunofluorescence staining

A list of the primary and secondary antibodies used for immunofluorescence staining, their dilutions and in which tissue sample they were used. R – Rabbit, D – Donkey, H – Human, and M – Mouse.

Human and mouse tissue sections stored at -80˚C were air dried at room temperature for 15 minutes and fixed with ice cold acetone for a further 10 minutes. After fixation, the slides were left to dry at room temperature for 20

minutes; during this time a circle enclosing the sections was drawn with a PAP pen (Dako, UK.) a hydrophobic marker which maintains any solution within the confined space created. The slides were then washed in 1X PBS solution for a duration of 3 minutes; this washing process was repeated three times. The sections were blocked using 10% donkey serum (Sigma) in PBS and left at room temperature for 20 minutes to block non-specific binding. Without washing, the blocking solution was tapped off and 20µl of the primary antibody was added to each individual section; parafilm was cut into roughly 1x0.8cm sections and placed over the tissue sections to disperse the antibody solution evenly across the entire section. The concentration of the primary antibody used was first determined by a series of small dilution ratios in PBS. Once the primary antibody concentration was determined (Table 2.3), it was added to the sections, covered in cut parafilm sections and left overnight at 4˚C in a humidified chamber. The negative controls were incubated in PBS instead of the primary antibody.

The parafilm sections were removed and the slides were washed thoroughly to remove the primary antibody by immersion in 1x PBS for 5 minutes, repeating the process three times. 20µl of the secondary antibody was added (see Table 2.3); again sections of parafilm were placed over the tissue section. For a second negative control, PBS was added to the section. The slides were incubated for one hour at room temperature to allow binding to occur, followed by a washing step in 1x PBS for 5 minutes repeated four times. Slides were mounted with a cover slip using Vectashield with DAPI (VWR) as a mounting medium.

2.2.5 Microscopy

2.2.5.1 Fluorescent microscopy

Using the fluorescent microscope (Nikon, Tokyo, Japan), sections either stained with SACPIC or fluorescent antibodies were placed onto the microscope and using the ACT-2U image analysis software, images were captured at different magnifications.

Sections stained with SACPIC; two for each IAP and their antagonist for matching terminal and vellus hair bearing skin from three different donors, using only the light with no filters, images of the skin and hair follicle were taken.

For sections stained with fluorescent antibodies and DAPI, an image of a section of interest was taken, and without dislodging the slide, different filters were put into place to capture FITC and DAPI.

2.2.5.2 Confocal microscopy

Once staining was completed, slides were placed in Zeiss Axioert200M microscope and using the Software (LSM510), images at different magnification were taken. Specific sections including the epidermis, dermis and hair follicle structures (if present) were taken for analysis. Two sections of each sample were taken for each individual donor, and three donors were analysed comparing terminal hair bearing skin to vellus hair bearing skin. Setting for the confocal microscope was saved for each objective magnification (adjusted eye pin hole and fluorescent gain) and the same setting was used for each individual section taken to allow not only for localisation within the section, but also for the comparison of fluorescent intensity between the different sections to compare between different proteins and skin type. A scale of 0-4 was used to identify the intensity of the images, with 0 being not expressed, to 4 being extremely highly expressed; all analysis was carried out by the same person.

2.2.6 Statistics

For the significance between the epidermal thickness between terminal and vellus hair bearing skin, an un-paired Students T-test of unequal variance was conducted.

2.3 Results

2.3.1 Localisation of IAPs in the mouse skin

Extensive studies have been carried out on the mouse hair cycle stage to identify the specific phases from the structure of the hair follicle (Muller-Rover et al., 2001). Another method to identify the stages of the hair cycle in specific breeds of mice was to observe its skin colour. Skin turns grey at mid anagen, and by late anagen the skin of the mouse is black, returning back to grey at catagen then pink when hair follicles are in telogen. This is because in these mice, melanocytes are only found in the hair follicle; in telogen, the melanocytes are reduced and inactive.

From the guideline below, sections from day 3 (early anagen), 5 (mid anagen), 12 (late anagen), 19 (catagen) and 21 (telogen) after depilation were used for analysis. Depilating hair follicles from the skin induced wounding (Xie et al., 2012); since the hair follicles were in a sense ripped from their natural environment causing an inflammatory response associated with wounding (Benavides et al., 2009). Therefore, results obtained from the different stages may also reflect changes in IAPs in response to the wound healing response of the hair follicle and surrounding dermis.

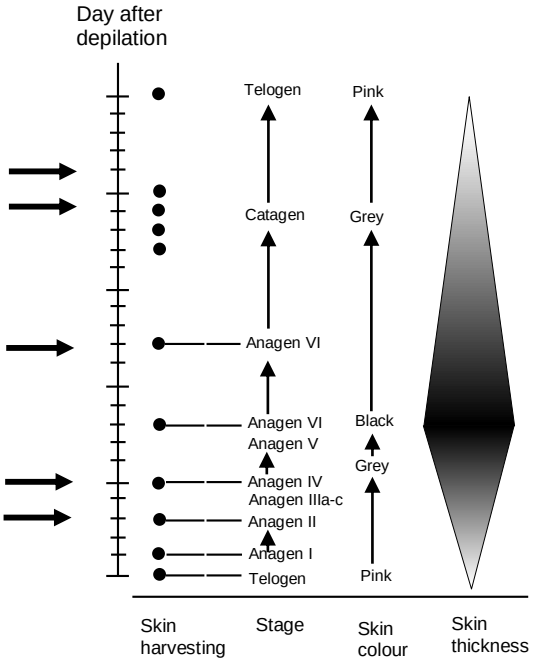


Figure 2.3: Hair cycle stages for mouse hair follicles after depilation

Guideline to hair follicle thickness at different stages of the hair cycle at different days after depilation. At roughly day 3 after depilation, the hair follicle should be in early anagen, at day 5 after depilation, the hair follicle should be at mid anagen, at 12 days after depilation, the hair follicle should be at late anagen. At day 19 after depilation, the hair follicle should have gone into the catagen transition, and from day 21 onwards, the hair follicle is entering telogen. Arrows indicate time points which skin was harvested (Muller-Rover et al., 2001).

2.3.1.1 Apollon (BIRC6)

Apollon was highly expressed in the epidermis of the mouse skin following depilation (Figure 2.4A-F). Decreasing at day 12 to 19, which corresponds to the anagen-catagen transition phase of the hair cycle and increasing again at day 21. A similar pattern of expression was seen throughout the hair cycle in the outer root sheath of the hair follicle. Expression of Apollon in the bulge region remained high throughout the hair cycle except at day 19, corresponding with catagen, when expression could not be detected. Low to no expression of Apollon was seen in the dermal papilla throughout the hair cycle. Low levels of Apollon was expressed in the fibroblasts in the dermis at day 5 and 21 after depilation. At day 3, 12 and 19 after depilation, Apollon was not detected in the cells of the dermis. Cellular staining of Apollon was cytoplasmic. A summary of the expression of Apollon is given in Table 2.4.

2.3.1.2 cIAP1 (BIRC2)

cIAP1 was detected in the epidermis of murine skin throughout the stages of the hair cycle (Figure 2.4G-K); its expression was high at day 3 and 5 after depilation corresponding with the early stages of anagen. Expression decreased 12 and 19 days after depilation, with expression lowest 21 days after depilation. It was expressed in all the layers of the epidermis. cIAP1 was expressed in the dermal fibroblasts in the dermis surrounding the hair follicle, and expression remained high until day 21. Expression in the outer root sheath of the hair follicle gradually decreased; high expression was detected 3 and 5 days after depilation, with a decreased expression 12 and 19 days after depilation; a further decrease of cIAP1 was observed at day 21. Expression at the inner root sheath was constant through the hair cycle except for day 3 and 21 after depilation where it was not observed. At day 3 after depilation, moderate expression of cIAP1 was seen in the dermal papilla, with no expression after 5, 12 or 19 days. Low levels of expression were detected at 21 days after depilation. cIAP1 staining was located in the cytoplasm of the cells. A summary of the expression of cIAP1 is given in Table 2.4.

2.3.1.3 cIAP2 (BIRC3)

cIAP2 was clearly expressed in the epidermis of murine skin throughout the hair cycle stages induced via depilation (Figure 2.4L-P), the expression was only detected in the basal layer, except at day 5 after depilation, where all the layers of the epidermis were stained. cIAP2 was not seen in the dermis throughout the hair cycle, with the exception of day 5 after depilation, where low levels were detected. It was highly expressed in the outer root sheath of the hair follicle throughout the hair cycle. However, cIAP2 was expressed in the inner root sheath only at day 5 after depilation; no expression was detected at other stages of the hair cycle. Expression was detected in the hair matrix at day 5 after depilation, at 12 days after depilation the expression of cIAP2 was located at one side of the matrix in a bilateral disc. Expression of cIAP2 19 days after depilation was detected in the dermal sheath surrounding the dermal papilla. cIAP2 was not expressed in the dermal papilla at any stage of the hair cycle. This protein was expressed in the cytoplasm of the cells. A summary of the expression of cIAP2 is given in Table 2.4.

2.3.1.4 XIAP (BIRC4)

XIAP was expressed in all layers of the epidermis of the mouse skin throughout the hair cycle (Figure 2.4Q-U), its expression was moderate 3 days after depilation, increasing 5 days after depilation, at day 12 expression decreased, remaining moderate at day 19 and 21 after depilation. Expression of XIAP in the dermis was only detected 5 days after depilation. Expression in the outer root sheath of the hair follicle was high at days 3 and 5, with reduced expression at day 12 and 19, its expression increased at day 21. Expression was moderate and constant in the inner root sheath at all stages of the hair cycle where it was present. No expression of XIAP was found in the dermal papilla throughout the hair cycle until 21 days after depilation when low expression was observed. XIAP was expressed in the cytoplasm of the cells. A summary of the expression of XIAP is given in Table 2.4.

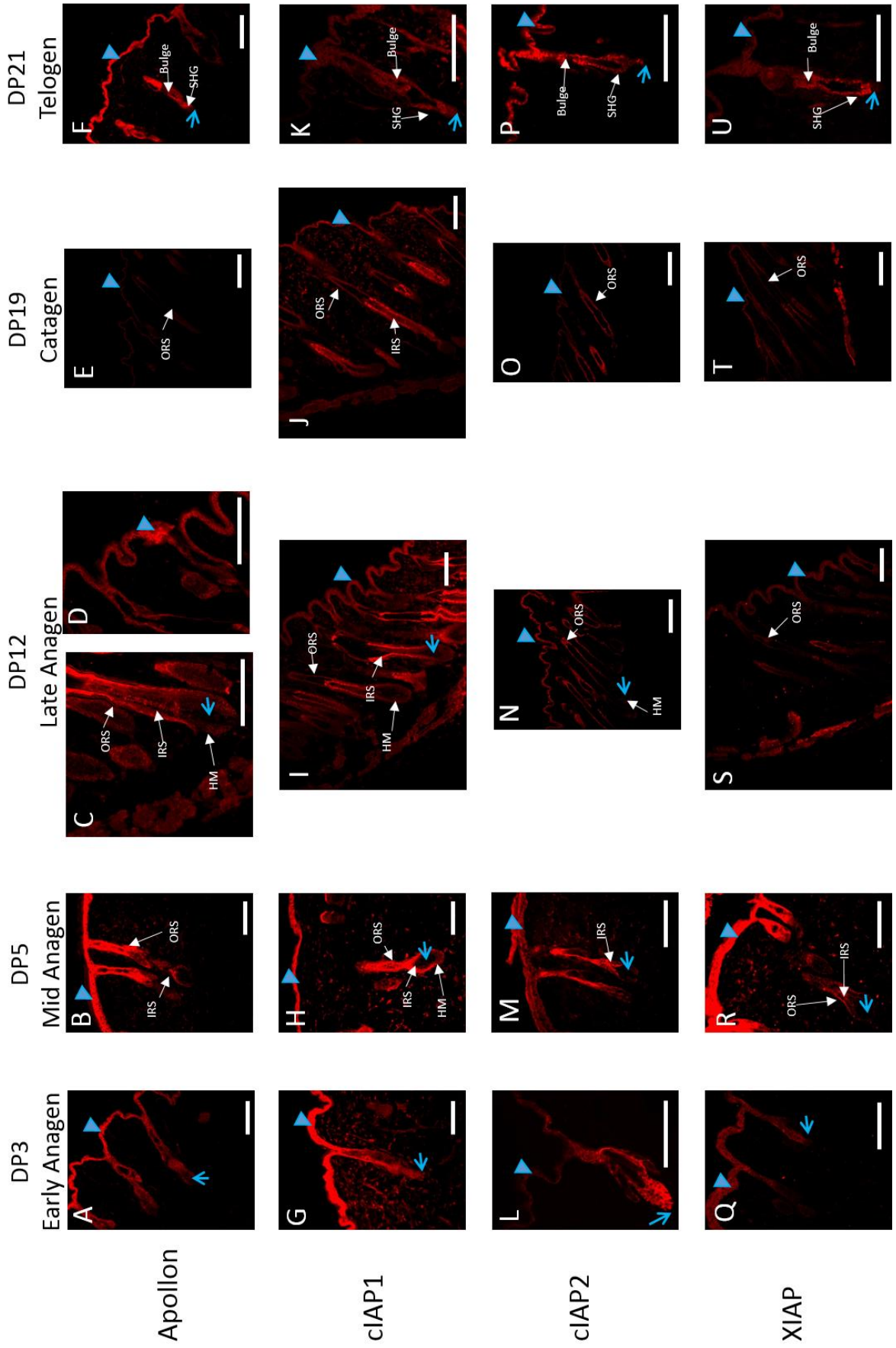

DP3
Early Anagen
DP5
Mid Anagen
DP12
Late Anagen
DP19
Catagen
DP21
Telogen
Apollon
cIAP1
cIAP2
XIAP
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
IRS
ORS
HM
Bulge
SHG

Figure 2.4: Expression of IAPs in C3H mice during different stages of the hair cycle induced by depilation

Immunofluorescence data representing the expression of IAPs Apollon, cIAP1, cIAP2 and XIAP at different stages of the mouse hair cycle induced by depilation. (A-F) Represents immunohistochemical expression of Apollon. A) Early anagen. B) Mid anagen. (C + D) Late anagen. E) Catagen. F) Telogen. (G-K) Represents expression of cIAP1. G) Early anagen. H) Mid anagen. I) Late anagen. J) Catagen. (L-P) Represents immunofluorescent expression of cIAP2. L) Early anagen. M) Mid anagen. N) Late anagen. O) Catagen. P) Telogen. (Q-U) Represents images for expression of XIAP. Q) Early anagen. R) Mid anagen. S) Late anagen. T) Catagen. U) Telogen. Representative image of n=2 sections. Blue arrow head – epidermis, blue arrow – dermal papilla, IRS – inner root sheath, ORS – outer root sheath, HM – hair matrix, SHG – secondary hair germ. Magnification bar 250µm.

Apollon		Skin		Hair Follicle					
Stage of Hair Cycle	Days after Depilation	Epidermis	Dermis	ORS	IRS	HM	DP	Bulge	SHG
Early Anagen	Day 3	+++	-	++			+	++	
Mid Anagen	Day 5	+++	+	+++	++	++	-	+++	
Late Anagen	Day 12	++	-	++	+++	++	-	++	
Catagen	Day 19	+/-	-	+/-	+/-	+/-	-	-	
Telogen	Day 21	+++	+	++			+	+++	++

cIAP1		Skin		Hair Follicle					
Stage of Hair Cycle	Days after Depilation	Epidermis	Dermis	ORS	IRS	HM	DP	Bulge	SHG
Early Anagen	Day 3	+++	+++	+++			++	+++	
Mid Anagen	Day 5	+++	+++	+++	+++	++	-		
Late Anagen	Day 12	++	++	++	+++	++	-	+++	
Catagen	Day 19	++	+++	++	+++	+	-	+++	
Telogen	Day 21	+	+	+			+	++	++

cIAP2		Skin		Hair Follicle					
Stage of Hair Cycle	Days after Depilation	Epidermis	Dermis	ORS	IRS	HM	DP	Bulge	SHG
Early Anagen	Day 3	++	-	+++			-	+++	
Mid Anagen	Day 5	+++	+	+++	+++	+	-	+++	
Late Anagen	Day 12	+++	-	+++	-	++†	-	+++	
Catagen	Day 19	++	-	+++	-	++‡	-	+++	
Telogen	Day 21	+++	-	+++			-	+++	+++

XIAP		Skin		Hair Follicle					
Stage of Hair Cycle	Days after Depilation	Epidermis	Dermis	ORS	IRS	HM	DP	Bulge	SHG
Early Anagen	Day 3	++	-	++			-	++	
Mid Anagen	Day 5	+++	++	++	++	-	-		
Late Anagen	Day 12	++	-	+	++	+	-	+	
Catagen	Day 19	++	-	+	++	-	-	+	
Telogen	Day 21	++	-	++			+	++	+++

Table 2.4: Summary table of expression of IAPs in the mouse skin and hair follicle

IAP expression in the skin and hair follicle of C3H mice at different time points following depilation. Day 3 – early anagen, day 5 – mid anagen, day 12 – late anagen, day 19 – catagen, day 21 - telogen. ORS – outer root sheath, IRS – inner root sheath, HM – hair matrix, DP – dermal papilla, SHG – secondary hair germ. Average expression from 2 sections. (blank) not observed, - no expression, + low expression, ++ moderate expression, +++ high expression. † *- partial expression in a bilateral expression, on one side of the hair matrix,* ‡ *- surrounding the dermal papilla.*

2.3.2 Localisation of IAP antagonists in the mouse skin

2.3.2.1 DIABLO

DIABLO was expressed in all compartments of murine skin and hair follicles during the hair follicle cycle (Figure 2.5A-G). Expression was detected in all layers of the epidermis and did not change. In the dermis, expression was moderate and constant throughout the hair cycle. In the hair follicle compartments, DIABLO was expressed in the outer and inner root sheath at a moderate level throughout the hair cycle, increasing slightly at day 19, during catagen. Moderate expression was found in the dermal papilla 3 and 5 days after depilation, decreasing slightly at day 12 after depilation with a slight granular staining appearance. Expression increased 19 days after depilation, reducing at day 21. Low levels of DIABLO was expressed in the bulge region, with a slight increase at day 21 after depilation. Low expression of DIABLO was detected in the hair matrix at day 5 and 12 after depilation, with an increase at day 19. Expression of DIABLO was cytoplasmic in cells. A summary of the expression of DIABLO is given in Table 2.5.

2.3.2.2 Xaf1

Xaf1 was expressed in the epidermis and dermis of the mouse skin throughout the hair cycle stages induced via depilation (Figure 2.5H-L). Its expression in the epidermis was moderate to high throughout the hair cycle. Expression of XAF1 in the dermis was moderate to high from day 3 to 5 after depilation. Expression was not seen at day 12, with a slight increase in Xaf1 expression at day 19 and 21 after depilation. High expression was seen in the outer root sheath throughout the hair cycle with the exception of day 12 after depilation, where expression decreased. Moderate to high expression was seen in the inner root sheath from day 3 to day 12 following depilation. Low to no expression was seen in the dermal papilla throughout the hair follicle cycle, with a slight increase at day 21. This expression of XAF1 in cells was cytoplasmic. A summary of the expression of Xaf1 is given in Table 2.5.

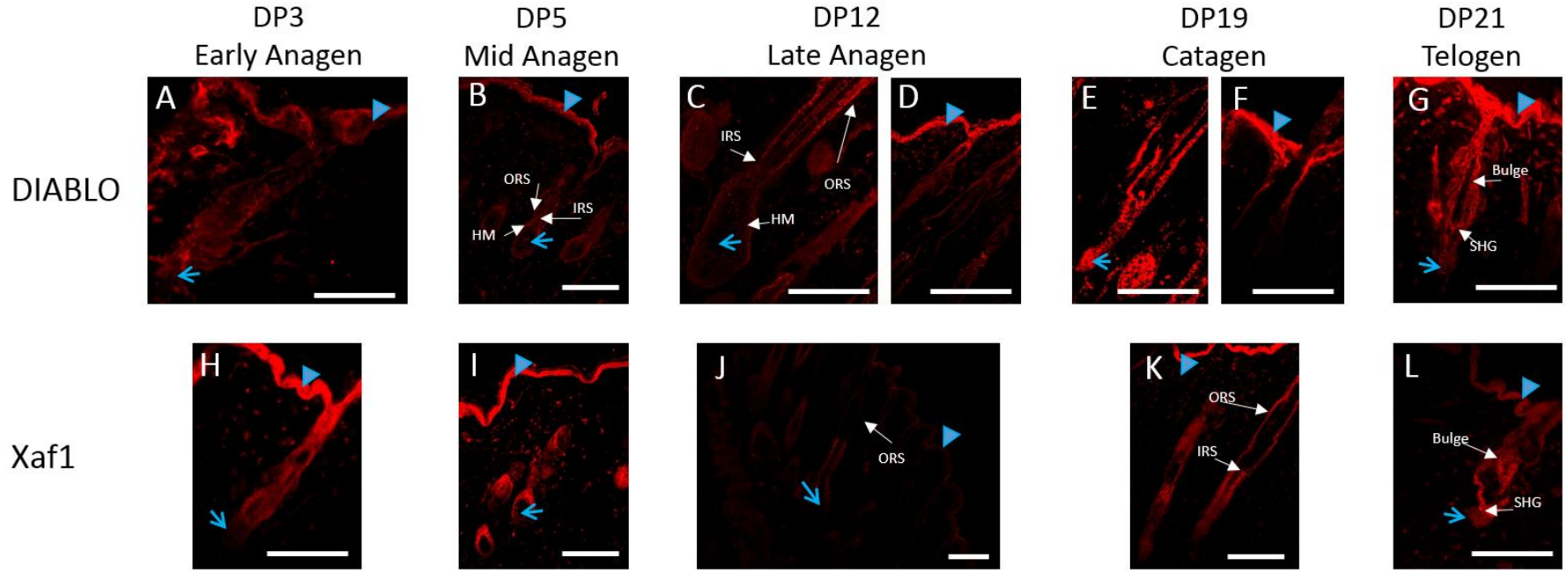


Figure 2.5: Expression of the IAP antagonist DIABLO and Xaf1 detected in all stages of the hair cycle in the epidermis, dermis and hair follicle

Immunofluorescence images for DIABLO and Xaf1 expression throughout the hair cycle induced by depilation. (A-G) Expression of DIABLO in the hair follicle cycle. A) Early anagen. B) Mid anagen. (C + D) Late anagen. (E + F) Catagen. G) Telogen. (H-L) Expression of Xaf1 in the hair follicle cycle. H) Early anagen. I) Mid anagen. J) Late anagen. K) Catagen. L) Telogen.

Blue arrow head – epidermis, blue arrow – dermal papilla, IRS – inner root sheath, ORS – outer root sheath, HM – hair matrix, SHG – secondary hair germ. Magnification bar 250µm

DIABLO	Skin		Hair Follicle					
Days after Depilation	Epidermis	Dermis	ORS	IRS	HM	DP	Bulge	SHG
Day 3	++	++	++			++	+	
Day 5	+++	++	++	++	+	++		
Day 12	+++	++	++	++	+	+	+	
Day 19	+++	++	+++	+++	+++	+++	+	
Day 21	+++	++	++			++	++	++

Xaf1	Skin		Hair Follicle					
Days after Depilation	Epidermis	Dermis	ORS	IRS	HM	DP	Bulge	SHG
Day 3	+++	++	+++			+	+++	
Day 5	+++	+++	+++	+++	+	-	+++	
Day 12	++	-	+	++	+	-	+	
Day 19	+++	+	+++	+++	+	+	+++	
Day 21	++	+	++			++	++	+++

Table 2.5: Summary table of expression of IAP antagonists in the mouse skin and hair follicle

Overview of the IAP antagonists DIABLO and Xaf1 expression in murine skin and hair follicles at different time points after depilation. *Day 3 – early anagen, day 5 – mid anagen, day 12 – late anagen, day 19 – catagen, day 21 - telogen*. ORS – outer root sheath, IRS – inner root sheath, HM – hair matrix, DP – dermal papilla, SHG – secondary hair germ, Average *expression from 2 sections*. (blank) not observed, - no expression, + low expression, ++ moderate expression, +++ high expression.

2.3.3 Human terminal and vellus hair follicle histology

The human hair follicle cycle is not synchronised, and for *in vivo* studies, cannot be depilated to get uniformed hair follicle growth. Most hair follicles in the scalp are in anagen, but to clearly determine the stages of the hair cycle SACPIC staining was used; this is a useful technique since staining multiple components (Table 2.6) on the same section can be achieved to differentiate between hair follicle stages (Nixon, 1993).

Colour:	Sections:
Blue	Collagen
Green	Smooth muscle
Blue/Pink	Undifferentiated keratinocytes
Red	Keratinising inner root sheath
Blue/Grey	Pre-keratinising inner root sheath
Orange/Brown	Trichohyalin granules
Blue	Outer root sheath
Pale Blue	Connective tissue sheath
Yellow	Keratinising hair fibre
Pink	Pre-keratinising hair fibres
Pale Blue	Dermal papilla extra cellular matrix
Blue/Black	Dermal papilla cells
Orange/Yellow	Outer borders of brush ends of catagen/telogen hair fibre
Dark Red	Stratum corneum
Dark Blue/Grey	Nuclei

Table 2.6: SACPIC staining key

Colour representation for SACPIC staining (Nutbrown and Randall, 1996).

Vellus hair follicles are also distinguished from terminal hair follicles by their bulb location in the dermis. Terminal hair follicle bulbs are located closer and into the hypodermis, whilst vellus hair follicles are closer to the epidermis. The size of the hair follicles can also help to differentiate between the two types of hair follicles (Garcia et al., 2011), the length of vellus hair shaft are no longer than 2mm, whilst in terminal it is longer than 2mm, vellus hair thickness is roughly 30µm compared to 60µm in terminal (Garcia et al., 2011)(Figure 2.6).

SACPIC staining demonstrated that the hair follicles from the human terminal hair bearing scalp skin were in anagen. There was a slight red staining indicating the keratinising inner root sheath. The yellow indicated the keratinising hair fibre. Blue staining represents the outer root sheath. The hair follicles are surrounded by blue staining indicating the collagen in the dermis. The bulb region was distinctive with a clear dermal papilla compartment surrounded by the hair matrix, enclosed by the dermal sheath. The bulb region was closer to the hypodermis than the epidermis (Figure 2.6A).

A representation of a vellus hair follicles from human facial skin appeared to be in the catagen/telogen transition due to a brush like appearance at the shaft beginning, and the morphology of the bulb region (Figure 2.6C). The hair fibre was stained yellow and the outer root sheath blue, pale blue staining around the hair follicle represents the dermal tissue sheath. The vellus hair follicle can be distinguished from the terminal hair follicle from the size and the location of the bulb region. The bulb region of vellus hair follicles does not distend beyond the sebaceous gland; the diameter of the hair follicle is also smaller (Figure 2.6B). An epithelial stalk with the dermal papilla at the end indicates that the hair follicle was in late catagen/telogen transition.

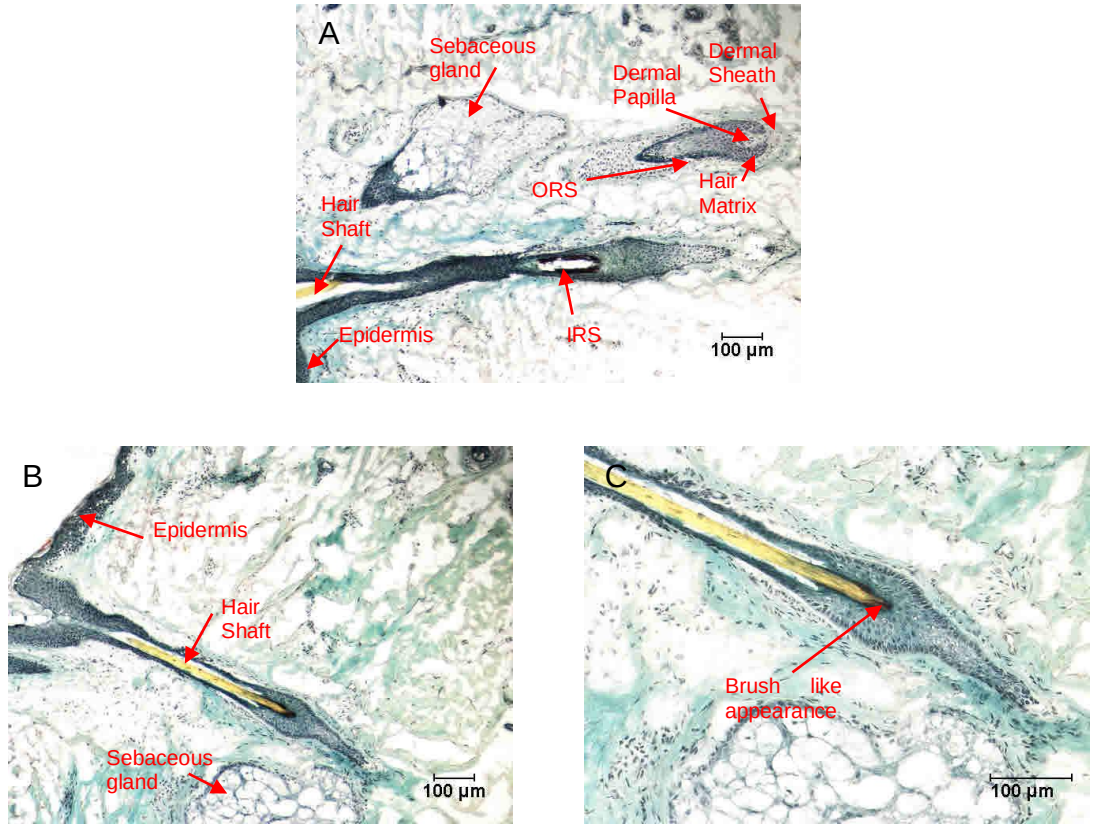


Figure 2.6: SACPIC staining of human skin sections from female scalp containing terminal hair and vellus hair follicles

SACPIC staining to distinguish hair follicle from anagen and telogen stages of the hair cycle in terminal and vellus hair bearing skin. A) SACPIC staining from a terminal hair bearing skin. B and C) Represent SACPIC staining for vellus hair follicles at x20 and x40 objective magnification respectively. Magnification bar 100µm.

2.3.4 Epidermal thickness in vellus hair bearing skin is thinner compared to matching terminal hair bearing skin

To determine differences in epidermal thickness between terminal and vellus hair bearing skin, thickness was measured from three matching donors by calculating pixel length. For each donor, epidermal thickness was normalised against the thickness from terminal hair bearing skin (Figure 2.7). Epidermal thickness from vellus hair bearing skin (~66.85µm) was significantly thinner than corresponding terminal hair bearing skin (~82.99µm) ($p<0.05$), Student T-test.

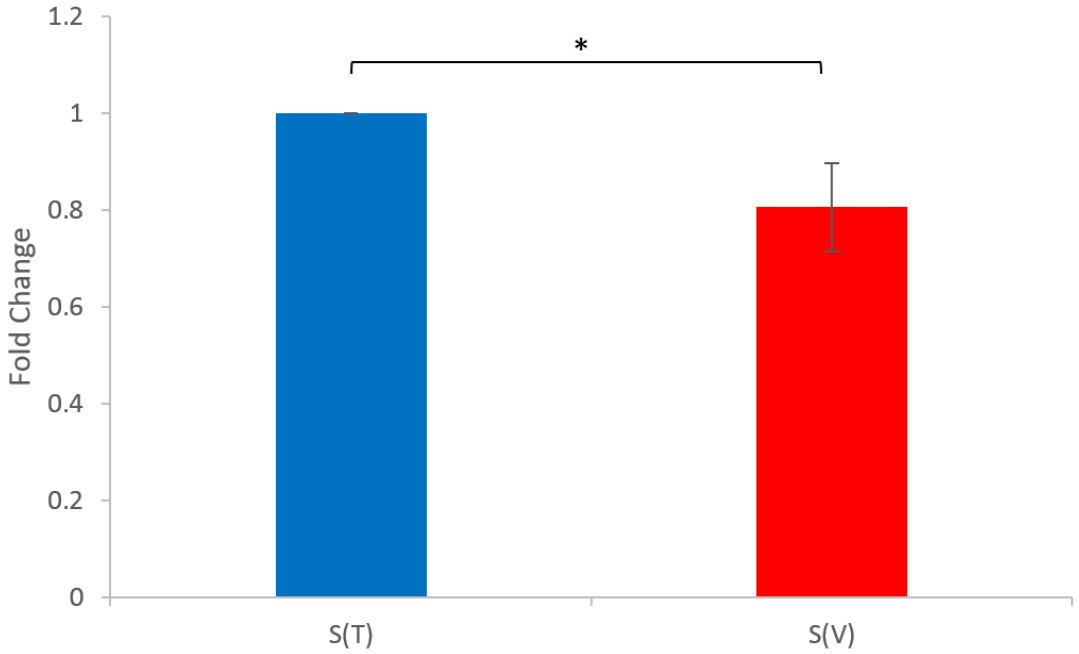


Figure 2.7: Epidermal thickness from vellus hair bearing skin was thinner than matching terminal hair bearing skin

*A comparison of epidermal thickness between terminal hair bearing skin – S(T) (blue) and vellus hair bearing skin – S(V) (red) from three matching donors normalised against the thickness from terminal hair bearing skin. * $p<0.05$, Student T-test.*

2.3.5 Localisation of IAPs in the human terminal and vellus hair follicle bearing skin

Expression and localisation of IAPs and their antagonists in terminal and vellus hair bearing skin was determined via immunofluorescent staining on three matching donor samples (Figure 2.8 and Figure 2.9 respectively)

2.3.5.1 Apollon (BIRC6)

Apollon was expressed (Figure 2.8A-E) at moderate to high levels in the epidermis of vellus hair bearing skin. Its expression was seen in all layers of the epidermis (Figure 2.8A). Apollon was also detected in fibroblasts and the ECM in the dermal layer of the skin. A similar level of expression was seen in terminal hair bearing skin, in both the epidermis and dermis (Figure 2.8C).

In the vellus telogen hair follicle, Apollon was not expressed in the dermal papilla, but expressed at moderate levels in the outer root sheath (Figure 2.8B).

In the hair follicle compartments, high expression of Apollon was detected in the inner and outer root sheath of the terminal hair follicle (Figure 2.8D). In the bulb region of the hair follicle, Apollon was expressed in the hair matrix and dermal sheath, with low to moderate levels seen in the dermal papilla (Figure 2.8E). A summary of the expression of Apollon is given in Table 2.7A.

2.3.5.2 cIAP2 (BIRC3)

Expression of cIAP2 (Figure 2.8F-J) in the epidermis of vellus hair bearing skin (Figure 2.8F) was lower than that in the epidermis of corresponding terminal hair bearing skin from the same donor (Figure 2.8I). Likewise, lower expression was also seen in the dermis of vellus hair bearing skin compared to corresponding terminal hair bearing skin.

Vellus hair follicles expressed lower levels of cIAP2 in the inner and outer root sheath and there was no expression in the dermal papilla and hair matrix cells

of the bulb (Figure 2.8G); there was also no expression of cIAP2 in the mesenchymal cells of the dermal sheath in vellus hair follicles.

In the anagen terminal hair follicle, higher expression of cIAP2 was seen in all regions (Figure 2.8I and J) than corresponding vellus hair follicles (Figure 2.8G). A summary of the expression of cIAP2 is given in Table 2.7B.

2.3.5.3 NAIP (BIRC1)

Similar levels of NAIP expression (Figure 2.8K-O) were seen in the epidermis and dermis of both terminal (Figure 2.8K) and vellus (Figure 2.8M) hair bearing skin.

Vellus hair follicles displayed moderate to high levels of expression of NAIP in the inner root sheath, the outer root sheath and the dermal sheath. However, there were only low levels of expression in the hair matrix, with no expression in the dermal papilla (Figure 2.8L).

In contrast, the terminal hair follicles displayed little to no expression of NAIP in the inner root sheath, with moderate expression in the outer root sheath and the dermal sheath (Figure 2.8N). In the bulb, moderate expression of NAIP was seen in the hair matrix with low levels of expression in the dermal papilla (Figure 2.8O). A summary of the expression of NAIP is given in Table 2.7C.

2.3.5.4 XIAP (BIRC4)

A similar level of XIAP expression (Figure 2.8P-T) was seen in the epidermis and dermis of vellus hair bearing skin (Figure 2.8P) and corresponding terminal hair bearing skin (Figure 2.8R).

In the vellus hair follicle, XIAP was only expressed in low levels in the hair matrix and dermal sheath, it was not detected in other compartments of the vellus hair follicle (Figure 2.8Q).

In contrast, in the terminal hair follicle, moderate expression of XIAP was seen in the inner and outer root sheath (Figure 2.8S), hair matrix and dermal sheath (Figure 2.8T). A summary of the expression of XIAP is given in Table 2.7D.

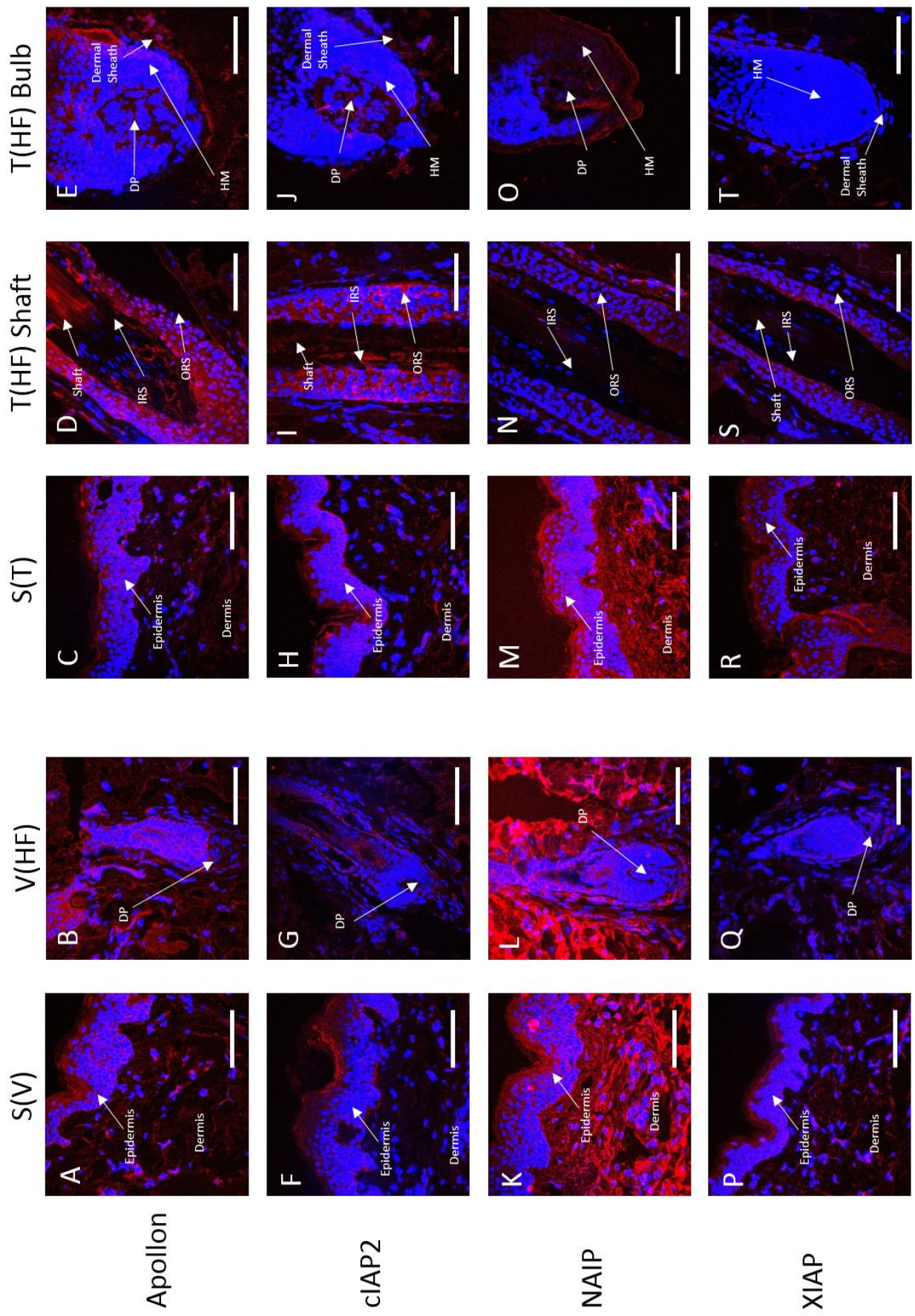

S(V)
V(HF)
S(T)
T(HF) Shaft
T(HF) Bulb
Apollon
cIAP2
NAIP
XIAP
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
Epidermis
Dermis
DP
Shaft
IRS
ORS
HM
Dermal Sheath

Figure 2.8: Expression of IAPs in vellus and terminal hair bearing skin, and hair follicle compartments.

Immunofluorescent expression of IAPs in the skin and hair follicle of human vellus and terminal hair bearing skin from the same donor. (A-E) Apollon. (F-J) cIAP2. (K-O) NAIP. (P-T) XIAP. (A, F, K, P) IAP expression in vellus hair bearing facial skin (S(V)). B, G, L, Q) IAP expression in vellus hair follicle (V(HF)) of vellus hair bearing facial skin. (C, H, M, R) IAP expression in terminal hair bearing scalp skin (S(T)). (D, I, N, S) IAP expression in the hair shaft region of terminal anagen hair follicles (T(HF)) in S(T). (E, J, O, T) IAP expression of the bulb region of T(HF) in S(T).

DP – dermal papilla, HM – hair matrix, IRS – inner root sheath, ORS – outer root sheath, red – IAP protein, blue DAPI – nucleus. Magnification bar 100µm.

A	Skin			Hair Follicle				
Apollon	EK	DF	ECM	IRS	ORS	HM	DP	DS
S(T)	+++	++	+++	++++	+++	+++	++	+++
S(V)	+++	+++	+++		++		-	++

B	Skin			Hair Follicle				
cIAP2	EK	DF	ECM	IRS	ORS	HM	DP	DS
S(T)	++	++	++	++	++	++	+	++
S(V)	+	+	+	+	+	-	-	-

C	Skin			Hair Follicle				
NAIP	EK	DF	ECM	IRS	ORS	HM	DP	DS
S(T)	+++	+++	++++	+	++	++	+	++
S(V)	++	+++	++++	++	+++	-/+	-	+++

D	Skin			Hair Follicle				
XIAP	EK	DF	ECM	IRS	ORS	HM	DP	DS
S(T)	++	+	++	++	++	++		++
S(V)	+	+	++		-		-	+

Table 2.7: Summary table of expression of IAPs in skin and hair follicle from vellus and terminal hair bearing skin

Summary of the IAP expression in the skin and hair follicle from vellus and terminal hair bearing skin. A) Apollon. B) cIAP2. C) NAIP. D) XIAP. S(T) – terminal hair bearing skin, S(V) – vellus hair bearing skin, EK – epidermal keratinocyte, DF – dermal fibroblast, ECM – extra cellular matrix, IRS – inner root sheath, ORS – outer root sheath, HM – hair matrix, DP – dermal papilla, DS – dermal sheath. Three matching patient section from terminal and vellus hair bearing skin were stained in duplicates, a value of 0-4 was used for each patient and each duplicate, an average was taken then rounded to full integer. - no expression, + low expression, ++ moderate expression, +++ high expression ++++ very high expression. Red represents expression for one donor sample.

2.3.6 A comparison of the expression of IAP antagonists in human terminal and vellus hair follicle bearing skin

2.3.6.1 DIABLO

Expression of DIABLO (Figure 2.9A-E) was high in the epidermis and dermis of vellus (Figure 2.9A) and terminal (Figure 2.9C) hair bearing skin from the same donor. Whilst expression of DIABLO was cytoplasmic in the epidermis of vellus hair bearing skin, it was both cytoplasmic and nuclear in terminal hair bearing skin. DIABLO expression levels were also high in the hair follicles (inner root sheath, outer root sheath, dermal papilla, hair matrix and dermal sheath) for both vellus (Figure 2.9B) and terminal (Figure 2.9D and E) hair follicles. A summary of the expression of DIABLO is given in Table 2.8A.

2.3.6.2 Xaf1

Expression of Xaf1 (Figure 2.9F-J) was moderate in the epidermis and dermis of vellus hair bearing skin (Figure 2.9F), with a slightly higher expression in corresponding terminal hair bearing skin (Figure 2.9H). High expression was seen in vellus hair follicles in the inner and outer root sheath, with moderate levels in the bulb (Figure 2.9G). Corresponding terminal hair follicles showed moderate expression in the inner and outer root sheath, with little or no expression in the bulb (Figure 2.9I and J). A summary of the expression of Xaf1 is given in Table 2.8B.

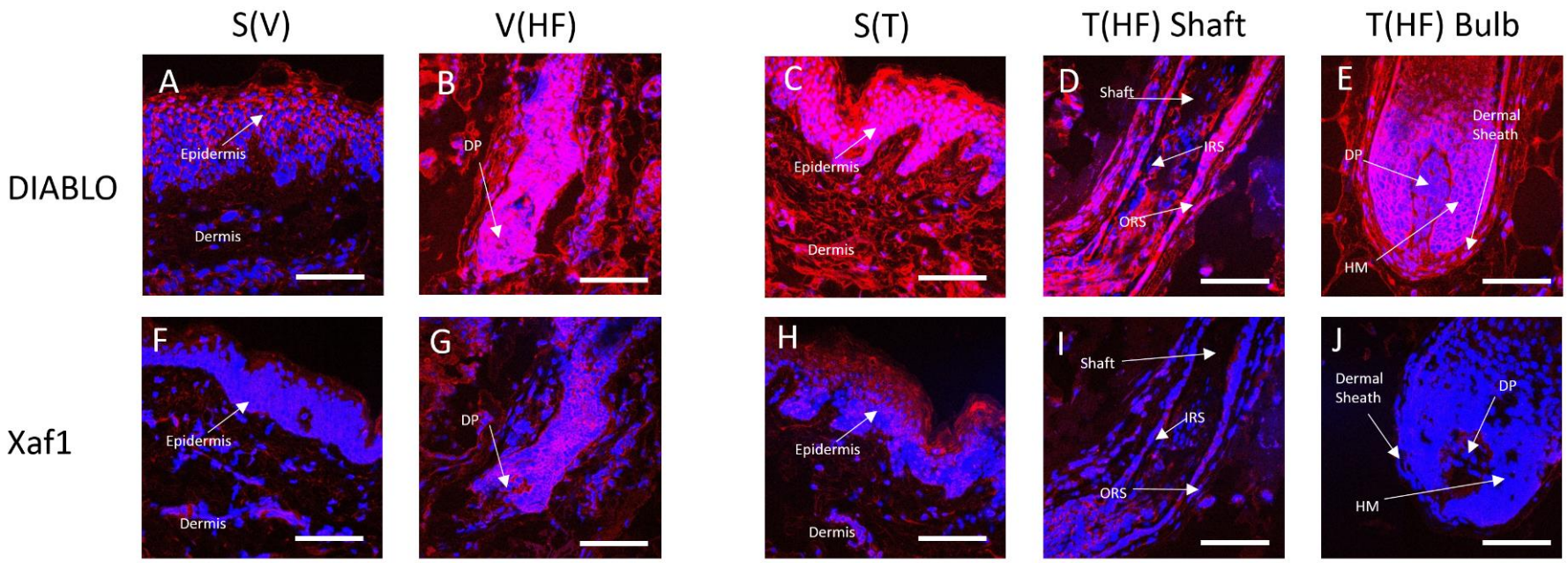


Figure 2.9: Expression of IAP antagonists in the skin and hair follicle compartments from vellus and terminal hair bearing skin.

Immunofluorescent expression of IAP antagonists in the skin and hair follicle of human vellus and terminal hair bearing skin from the same donor. (A-E) DIABLO. (F-J) Xaf1. (A and F), IAP antagonist expression in vellus hair bearing facial skin (S(V)). (B and G) IAP antagonist expression in vellus hair follicle (V(HF)) of vellus hair bearing facial skin. (C and H) IAP antagonist expression in terminal hair bearing scalp skin (S(T)). (D and I) IAP antagonist expression in the hair shaft region of terminal anagen hair follicles (T(HF)) in S(T). (E and J) IAP antagonist expression of the bulb region of T(HF) in S(T).

DP – dermal papilla, HM – hair matrix, IRS – inner root sheath, ORS – outer root sheath, red – IAP protein, blue DAPI – nucleus. Magnification bar 100µm.

A	Skin			Hair Follicle				
DIABLO	EK	DF	ECM	IRS	ORS	HM	DP	DS
S(T)	++++	++++	++++	++++	++++	++++	+++	++++
S(V)	++++	+++	+++	++++	++++	++++	++++	++++

B	Skin			Hair Follicle				
Xaf1	EK	DF	ECM	IRS	ORS	HM	DP	DS
S(T)	+++	+++	++	++	++	+	-/+	+
S(V)	++	++	++	+++	+++	++	++	++

Table 2.8: Summary table of expression of IAP antagonists in skin and hair follicle from vellus and terminal hair bearing skin

Summary of the IAP antagonist expression in the skin and hair follicle from vellus and terminal hair bearing skin. A) DIABLO. B) Xaf1. S(T) – terminal hair bearing skin, S(V) – vellus hair bearing skin, EK – epidermal keratinocyte, DF – dermal fibroblast, ECM – extra cellular matrix, IRS – inner root sheath, ORS – outer root sheath, HM – hair matrix, DP – dermal papilla, DS – dermal sheath. Three matching patient sections from terminal and vellus hair bearing skin were stained in duplicate, a value of 0-4 was used for each patient and each duplicate, an average was taken then rounded to full integer. - no expression, + low expression, ++ moderate expression, +++ high expression ++++ very high expression. Red represents expression for one donor sample.

2.4 Discussion

The hair follicle is an excellent model to study adult tissue regeneration. At the beginning of a new hair cycle, the lower portion of the hair follicle is remodelled, recapitulating events of late embryogenesis and high levels of mitotic activity in the epithelial matrix of the bulb (Commo and Bernard, 1997; Hashimoto et al., 2001; Tobin et al., 2003).

To study the hair cycle, it is important to use a model where the hair cycle is synchronised. The strongest known stimulus to induce the telogen (resting) to anagen (growing) switch, and produce synchronicity in growth is via depilation (Stenn et al., 1996; Stenn and Paus, 2001). The hair follicles will then all be at the same stage of the hair cycle after depilation, and can be followed through the whole cycle from anagen to telogen (Muller-Rover et al., 2001). However, depilation also induces a pronounced wound healing response of the hair follicle (Matsuo et al., 2003). Therefore, the mouse not only provides an appropriate model to follow changes in the expression of different IAPs and their antagonists throughout the hair cycle, since in a mouse model, the length of anagen is short (12-17 days) (Muller-Rover et al., 2001), but also to determine whether there are any changes in IAP expression induced by the wound healing response of the hair follicle following depilation.

2.4.1 IAP expression in murine skin and the hair follicle cycle

XIAP, cIAP1 and cIAP2 have been identified in mouse skin, mainly in the epidermis and hair follicles (Fessing et al., 2006; Seitz et al., 2000). However, to date, there have been no studies looking at the localisation of these proteins in the skin and hair follicle at different stages of the hair cycle. Depilation increases apoptosis in hair follicles and also induces a wound healing response to the tissue surrounding the hair follicle (Matsuo et al., 2003; Paus et al., 1998). Immunofluorescent staining has demonstrated that the IAPs XIAP, cIAP1, cIAP2 and Apollon are located in the skin and hair follicle of mice at all stages of the hair follicle cycle induced by depilation (Figure 2.4 and Table 2.4).

The expression of Apollon, cIAP1, cIAP2 and XIAP was seen predominantly in the epithelial cells of the epidermis and hair follicle (outer root sheath, inner root sheath and hair matrix). All were expressed in the bulge region of the outer root sheath, which contains the hair follicle stem cells (Ohyama, 2007). All were expressed in the secondary hair germ found only in telogen hair follicles (Ito et al., 2004). This indicates an importance for IAPs in the protection of stem cells; which are important in hair follicle regeneration and also epidermal regeneration following wounding. Double immunofluorescent staining for stem cells using specific markers such as Sox9 antibodies with IAP antibodies would establish whether stem cells express IAPs.

In contrast, with the exception of cIAP1, there was little expression in the dermis or mesenchymal-derived dermal papilla. cIAP1 was only expressed in the dermal papilla 3 days after depilation, corresponding to early anagen and then again in telogen at day 21 (Figure 2.4 and Table 2.4). Since the dermal papilla is a constant feature during hair follicle regeneration, it would be assumed that the levels of IAPs are highest and constant in the dermal papilla. Other inhibitors of apoptosis proteins such as Bcl2 are however found in the dermal papilla (Soma and Hibino, 2004). This indicates that IAPs may not have a predominant role in protecting the murine dermal papilla.

The expression of Apollon in both the epidermis and the hair follicle was reduced during the catagen stage of the hair cycle. Reduction in inhibitors of apoptosis proteins such as Bcl2 has been associated with the anagen-catagen transition (Botchkareva et al., 2006). Also the increase in caspase 3 and 8 activity in the outer root sheath of human hair follicles indicated that the hair follicle was in catagen (Botchkareva et al., 2006). Therefore, it was not surprising that the expression of Apollon decreased during this stage. Apollon has a unique method in preventing apoptosis, in that it degredates DIABLO, preventing it from binding to other IAPs (Hao et al., 2004). This would suggest that Apollon has an active role in preventing apoptosis in hair follicles and its down regulation may be important in the switch from anagen to catagen. With the reduced Apollon expression at day 19 after depilation, epidermal thickness

was also reduced, an indication that the hair follicle was in catagen (Muller-Rover et al., 2001). Reduced Apollon expression in the epidermis could indicate that Apollon may play a role in regulating skin thickness during catagen.

cIAP1 was the only IAP that was consistently expressed at high levels in the dermis (cellular and ECM). In early anagen, expression was mainly cellular and some expression was detected in the ECM, however, 5 days after depilation until telogen, expression was exclusively cellular. However, the level of expression in the epidermis and dermis was reduced when the hair follicle was in telogen, but remained moderate in the bulge and secondary hair germ indicating that cIAP2 may have a protective function on hair follicle stem cells, which are needed for the regeneration of the hair follicle in anagen (Myung and Ito, 2012). Its low expression in the epidermis and dermis could indicate that at this stage of the hair cycle, cIAP1 does not play a role in the epidermis. At 3 days after depilation, expression of cIAP1 was high in the epidermis and dermis, and also in the dermal papilla when the hair follicle was in early anagen. At early anagen, the dermal papilla signals to the surrounding hair epithelial cells to initiate the growth phase (Chi et al., 2010). Although it is important to protect the dermal papilla at all stages of the hair cycle (consistent cells at every hair cycle) (Soma and Hibino, 2004), during catagen and telogen, the dermal papilla is quiescent (Botchkarev and Kishimoto, 2003). Since cIAP1 expression was not expressed in the dermal papilla in late anagen and catagen, its expression could be related to the dermal papilla's quiescent state; regulating dermal papilla function. Its expression in the dermal papilla in early anagen could be in response to depilation and the pronounced wound healing response triggered (Matsuo et al., 2003), as a backup mechanism to Bcl2. Apoptosis increases following depilation, due to local tissue damage; an increase in cIAP1 in the dermal papilla, could provide a mechanism to ensure its protection. To clarify this, it would be important to compare cIAP1 expression in depilated induced early anagen, and naturally occurring early anagen in the spontaneous hair cycle. The first post natal hair

cycle would need to be studied, as the first hair follicle cycle is synchronised without depilation, with subsequent hair cycles being less synchronised (Muller-Rover et al., 2001). No studies have looked at the effect of wounding on cIAP1 levels in skin and hair follicles.

The expression of cIAP2 was not dissimilar to Apollon in the epidermis and dermis (Figure 2.4 and Table 2.4). However, whilst the expression for Apollon in the outer root sheath decreased during catagen, cIAP2 expression remained constant throughout the hair cycle. In the bulge, cIAP2 expression was high and constant throughout the hair cycle, which could indicate that cIAP2 is important in protecting the stem cell niche, which is tightly regulated in hair follicle regeneration (Myung and Ito, 2012). cIAP2 expression was only seen 5 days after depilation in the inner root sheath; during late anagen and catagen, expression of cIAP2 was not present. In late anagen and catagen, the inner root sheath undergoes apoptosis (Magerl et al., 2001). This could indicate that the loss of cIAP2 expression regulates apoptosis in the inner root sheath. The lack of expression in the dermal papilla could indicate that this IAP does not interact and function to protect the dermal papilla from apoptosis. Its expression in the hair matrix in late anagen had a similar pattern of expression to Edar (Fessing et al., 2006). Defects in the gene encoding for Edar causes hypohidrotic ectodermal dysplasia which is characterised by a defect in the development of skin appendages such as the hair follicle (Azeem et al., 2009; Monreal et al., 1999). Edar has been associated with XIAP expression in the catagen hair follicle preventing apoptosis by positively regulating XIAP, in Edar deficient mice, there was an early onset and accelerated progression of catagen. It was therefore hypothesised that Edar regulates catagen development via XIAP regulation. Expression of cIAP2 in the hair matrix was in a unilateral pattern, similar to that of Edar, which could suggest that either Edar also regulates cIAP2 in a similar fashion to XIAP, or that cIAP2 controls Edar signalling, which in turn regulates XIAP expression, controlling catagen progression. A double immunostaining would need to be carried out to ensure that Edar positive cells also express cIAP2, and whether

the knockdown or down regulation of either Edar or cIAP2 has an effect on the other. Another hypothesis suggested by Lavker et al. (2003), was that the unilateral expression are segments derived from the hair bulge that have migrated to the hair matrix. Expression of bulge stem cell markers using double immunofluorescent staining would establish whether the cells expressing cIAP2 in the hair matrix are derived from the bulge stem cell niche. These cells are important in the regeneration of the hair follicle, which would suggest a role for cIAP2 in hair follicle regeneration.

The expression of XIAP in the epidermis was similar to Apollon, cIAP1 and cIAP2. It was not expressed in the dermis except at day 5 after depilation similar to Apollon and cIAP2 (Figure 2.4 and Table 2.4). The collaborative expression of these IAPs at 5 days after depilation, would indicate that either the IAPs were responding to the wound induced by depilation, ensuring that only the damaged cells were removed, with a higher expression at this time point to ensure no further apoptosis occurs, or that the IAPs regulate this stage of the hair cycle, and that the increase in IAP expression triggers the hair follicle to enter into catagen. In a similar experimental design to that of Fessing et al. (2006), knockout of specific IAPs in mice could determine the effect these IAPs have on hair follicles, and whether the knockout of cIAP2 has an effect on the hair cycle especially inducing catagen. Fuchs et al. (2013) showed that in wild type mice, XIAP is expressed at low levels in the bulge region of telogen hair follicles. Similar observations were seen in this study, showing low to moderate expression of XIAP in the bulge at all stages of the hair cycle. The study by Fuchs et al. (2013) did not look at the expression of XIAP throughout the hair cycle, and no other study has shown the effect of XIAP on hair follicle cycling. However, using similar methods of XIAP knockout, hair follicle cycling can be analysed to determine the importance of XIAP.

2.4.2 IAP antagonist expression in murine skin and the hair follicle cycle

DIABLO is the antagonist to all IAPs, its function either binds directly to IAPs, or it prevents the interaction of IAPs with caspases, allowing the caspase

cascade to occur (Verhagen et al., 2000). In contrast, Xaf1 is a direct and specific XIAP antagonist (Liston et al., 2001), binding to XIAP preventing its interaction with caspases.

Interestingly, both DIABLO and Xaf1 were expressed in the epidermis throughout the hair cycle. DIABLO was also constantly expressed in the dermis, while Xaf1 was highly expressed 5 days after depilation, with no expression at day 12 and low expression 19 and 21 days after depilation. The expression of Xaf1 mirrored the expression of XIAP (see Figure 2.4/Table 2.4 and Figure 2.5/Table 2.5).

In the rat endometrium, the expression of XIAP was the opposite to that of DIABLO during the estrus cycle (Leblanc et al., 2003); suggesting that DIABLO regulates XIAP expression; when the expression of DIABLO was high, the expression of XIAP was low, and when the expression of DIABLO was reduced, the expression of XIAP increased. The expression of XIAP was found at all stages of the estrous cycle (diestrus, proestrus, estrus and metestrus), but highest at estrus, while DIABLO expression was constant throughout the stages, except for estrus, which was significantly reduced. The administration of 17β-oestradiol to ovariectomised rats reduced DIBALO expression, and increased XIAP; suggesting that the regulation of XIAP via DIABLO is through 17β-oestradiol. However, the endogenous level of oestrogen peak during proestrus (Staley and Scharfman, 2005), but the biggest influence is during estrus which would explain the levels of XIAP and DIABLO. The study by Leblanc et al. (2003) supports that DIABLO regulates XIAP levels, however, since in this current study DIABLO expression was constant, it would suggest another role for DIABLO especially in the epidermis. A study by Murray et al. (2008) demonstrated that caspases may have another role aside from apoptosis, in their study, it was shown that caspase 3, regulated by caspase 9 was important in the differentiation of muscle cells (myoblasts to myotubes). Keratinocyte proliferation and differentiation is tightly controlled in the epidermis (Gandarillas, 2000), therefore since DIABLO interacts with caspase 9, it is possible that in the epidermis, DIABLO regulates

keratinocyte differentiation throughout the different layers. In the dermis, expression was moderate throughout the hair follicle cycle, a study by Liu et al. (2013) compared the expression of DIABLO in fibroblasts derived from normal and hypertrophic scars (excessive fibroblast and collagen production); DIABLO expression was reduced in hypertrophic scar fibroblasts. When Liu et al. (2013) studied proliferation of hypertrophic scar fibroblasts, they showed that over expression of DIABLO reduced proliferation. Therefore in this study, expression of DIABLO in the skin could be to regulate proliferation, since DIABLO expression was increased in the inner and outer root sheath, hair matrix and dermal papilla during catagen (apoptotic stage).

Xaf1 expression during catagen reflects that Xaf1 regulates XIAP. In the outer root sheath and inner root sheath where apoptosis is highest at day 19 after depilation, Xaf1 expression is high, while XIAP expression is low. Protection of stem cells in the bulge where stem cells reside (Ito et al., 2004) would be expected, however, XIAP expression is low, while Xaf1 expression is high. This could indicate that XIAP may not be important in hair follicle regeneration by protecting the stem cells.

The effect of hair follicle wounding on IAP and IAP antagonists is hard to analyse in this study. To ensure that the responses observed are solely due to hair follicle cycling or wound healing, it would be useful to compare their expression in mice undergoing their first natural hair follicle growth wave (Muller-Rover et al., 2001; Paus et al., 1999).

2.4.3 Expression of IAPs in human skin containing terminal or vellus hair follicles

In human scalp skin, 4mm biopsies from 31 Taiwanese donors showed that the total number of hair follicles from the scalp biopsies was 21.3, with 20.5 and 0.8 being terminal and vellus respectively (Ko et al., 2012); the area of the scalp is roughly 600cm^2 (Schwartz, 2005), therefore the total number of hair follicles in the scalp from the study by Ko et al. (2012) would be around roughly 31,950 hair follicles. The ratio of anagen to telogen was 91.6:8.4 and the ratio

of terminal to vellus was 25.3:1 (Ko et al., 2012). Similar results were seen in Texas donors where a 4mm scalp biopsy (region not mentioned) from 22 patients showed under normal conditions, the number of total hair follicles from the biopsies were 40, where 32.7 were terminal hair follicles in anagen, and 2.3 of the hair follicles were terminal hair follicles in telogen and 5 hair follicles were vellus, therefore the total number of hair follicles in the scalp would be around 60,000 hair follicles. The ratio of terminal to vellus was 7:1 and the ratio between anagen to telogen was 93.5:6.3 (Dy and Whiting, 2011; Lee et al., 2002; Whiting, 1993, 1996). In the study by Dy and Whiting (2011), there was a slightly higher ratio of terminal to vellus hair follicles in women compared to men, where in women the ratio was 11:1 and in men it was 6:1 in normal control donors. In this study, significance between male and female terminal:vellus ratio was not analysed.

In the present study, SACPIC staining was carried out on matching sections of skin containing terminal or vellus hair follicles to determine whether hair follicles were in the anagen or telogen phase. In this chapter, longitudinal sections were taken to analyse the type of hair follicle and the stage in which the hair follicle was in the hair cycle; therefore, limited data could be obtained analysing the expression of IAPs and their antagonists in human skin.

Measurement of epidermal thickness in terminal and corresponding vellus hair bearing skin showed a significantly thinner epidermis in vellus hair bearing skin compared to matching terminal hair bearing skin (Figure 2.7). A study by Vogt et al. (2007) compared hair follicle properties between terminal and vellus hair follicle obtained from scalp (region not specified) and retroauricular regions respectively. Thickness of the epidermis was thinner in vellus hair bearing skin compared to terminal hair bearing skin ranging from 64±12µm to 99±18µm in vellus and 72±16µm to 136±37µm in terminal hair bearing skin. Which corresponds with the results obtained in this study, indicating that hair follicles are important in epidermal thickness.

Unfortunately, studying the expression of proteins in human skin at different stages of the hair cycle to understand the implications of the hair cycle on IAP

expression is difficult, since the human hair cycle is not synchronised (Ebling, 1987), and impossible to synchronise in human donors unless depilated.

To date there has only been one study looking at the expression of XIAP, cIAP1 and cIAP2 in human skin and the hair follicle (Vischioni et al., 2006) with limited data as to which anatomical region the skin samples were taken and whether the samples were from male or female donors. This is the first study to compare the expression of NAIP, cIAP2, XIAP and Apollon in human skin containing terminal (scalp) and vellus (facial) hair from the same donor.

All IAPs (NAIP, cIAP2, XIAP and Apollon) were expressed in the epidermis, Apollon expression was similar in terminal and vellus hair bearing skin, whilst cIAP2, NAIP and XIAP showed higher epidermal expression in terminal hair bearing skin than corresponding vellus (Figure 2.8 and Table 2.7). Expression of these IAPs in the human epidermis correlated with those expressed in mouse epidermis at anagen.

The difference in IAP expression between terminal and vellus hair bearing epidermal skin could be due to the thickness of the epidermis. Vellus hair bearing epidermis was slightly thinner than terminal hair bearing epidermis (Figure 2.7).

While the expression of the IAPs NAIP, Apollon and XIAP was seen throughout the different layers of the epidermis, cIAP2 expression was higher in the basal layer of the epidermis. The basal layer of the epidermis is the only layer that is mitotically active, contributing to the formation of the ECM separating the epidermis from the dermis, mainly laminin5 by the secretion of integrins (α3β1) and laminin (Blanpain and Fuchs, 2006). Keratinocytes may also contribute to the ECM in the dermis via the upregulation and secretion of TGFβ1, which increases collagen synthesis and deposition from the fibroblasts (Wang et al., 2006). Also in the basal layer of the epidermis, in the rete ridges, stem cells are found (Blanpain and Fuchs, 2006; Lavker and Sun, 1982). This could indicate that cIAP2 may be important in protecting keratinocytes that are mitotically active and stem cell populations in the

epidermis. Expression of cIAP2 in HeLa cells (cervical cancer cell line) was increased during the G2/M stage of the cell cycle (Jin and Lee, 2006). Therefore expression of cIAP2 in the basal layer of the epidermis could relate to the mitotic stage of the keratinocyte.

In contrast to murine dermis, the IAPs NAIP, Apollon, cIAP2 and XIAP were expressed in the human dermis. Expression of cIAP2 and XIAP was low, while expression of NAIP and Apollon was high. Since the majority of the cells in the dermis are fibroblasts (Dams et al., 2011), which are important for ECM formation, NAIP and Apollon may be required for protecting and regulating dermal fibroblasts. No expression of these IAPs were found in the dermis of mouse skin at the anagen stage of the hair cycle. A study by Theerakittayakorn and Bunprasert (2011) compared fibroblasts from mouse (L929, cell line derived from C3H mice) and human (facial) skin. Morphologically, mouse dermal fibroblasts were smaller and proliferated faster than human dermal fibroblasts. Therefore these IAPs might decrease fibroblast proliferation, since they are not expressed in murine dermis but are expressed in human dermis. Further studies need to be conducted to determine whether this is indeed true by culturing dermal fibroblasts and blocking IAPs by using siRNA since individual IAP antagonists are not available to study individual IAP effects on proliferation.

Expression of these IAPs in the dermis was similar between vellus and terminal hair bearing skin, with the exception of cIAP2 which had a higher expression in the terminal hair bearing skin. The terminal hair follicles are in anagen, however, it is not clear what stage of anagen (early or late) it is, therefore it is not possible to know the effect of the hair follicle cycle on epidermis thickness.

Mouse skin was exposed to trauma with regards to depilation, IAP expression could have been low in the dermis of mice as a response to hair follicle wounding, which did not occur in human skin. Waxing, which was the method used to depilate mouse hair follicles (as opposed to plucking) causes an increase in immune cells (Ishimatsu-Tsuji et al., 2005; Sloat et al., 2012; Xiao

et al., 2012). cIAP1 was the only IAP that was expressed in the mouse dermis, which could be regulated due to the inflammatory response.

While no expression of NAIP was detected in mouse hair follicles (via PCR – data not published), this study showed that NAIP was present in human terminal and vellus hair follicles. This indicates that there are potentially different mechanisms in place between different species, reinforcing the importance of studying these proteins in humans.

Expression of the IAPs cIAP2, NAIP and XIAP were moderate in the terminal human anagen hair follicle with low expressions in the dermal papilla, while Apollon was highly expressed in the hair follicle with moderate expression in the dermal papilla. This is slightly different to mouse hair anagen hair follicles; since mouse anagen hair follicles did not express cIAP2, Apollon and XIAP in the dermal papilla. In contrast therefore, these IAPs may contribute to the protective state of the dermal papilla during the human hair cycle. Most studies have shown that it is Bcl2 that is highly expressed and contributes to dermal papilla protection during catagen in mouse and humans (Botchkareva et al., 2006; Lindner et al., 1997; Soma and Hibino, 2004), however, apoptosis regulation is complex and other proteins may also contribute to this function.

This is the first study to compare the expression of IAPs in terminal and vellus hair follicles from the same donor. However, while skin samples from three donors were analysed, vellus hair follicle was only obtained from one of these donors for immunofluorescent staining, making it difficult to compare the results between terminal an vellus hair follicles, also the vellus hair follicles were either anagen (for cIAP2 and NAIP staining) or telogen (for Apollon and XIAP staining). The expression of cIAP2, Apollon and XIAP was lower in the vellus outer root sheath, dermal papilla and dermal sheath expression compared to terminal hair follicles. A study by Miranda et al. (2010) demonstrated that intermediate hair follicles (which are follicles in the transition from vellus to terminal) were significantly smaller than terminal hair follicles. Therefore expression of IAPs could be due to follicle size.

2.4.4 Expression of IAP antagonists in human skin containing terminal or vellus hair follicles

DIABLO was highly expressed in both terminal and vellus hair bearing skin, throughout the epidermis, dermis and hair follicle including the dermal papilla (Figure 2.9 and Table 2.8). Its function is to promote apoptosis by binding to IAPs and allowing the caspase cascade to occur (Verhagen and Vaux, 2002). However, a difference in DIABLO expression between terminal and vellus hair bearing skin, is that the expression of DIABLO in vellus hair bearing skin was located in the cytoplasm of the keratinocytes, whilst in terminal hair bearing skin, it was located in both the cytoplasm and nuclei of keratinocytes. A study by Kim et al. (2006) showed that DIABLO translocated to the nucleus during the G2/M arrest resulting in apoptosis. In this study, they demonstrated that DIABLO interacts with Survivin at the mitotic spindle promoting apoptosis. The location of DIABLO in the nucleus would suggest that DIABLO is active since inactive DIABLO is found in the mitochondria (Adrain et al., 2001). This would suggest that in terminal hair bearing skin DIABLO was more active compared to vellus hair bearing skin. From the staining for the other IAPs, expression was not detected in the nucleus. It would be interesting to observe expression of keratinocyte cultures under wounding and non wounding conditions or other apoptotic stimuli such as UV, to establish whether IAPs translocate into the nucleus under stressed conditions, and whether expression of DIABLO is altered in the nucleus. Its location in the nucleus could indicate that DIABLO potentially regulates transcription factors. AP1 is an important transcription factor in the epidermis regulating epidermal differentiation (Eckert et al., 2013). To determine whether DIABLO does indeed interact with AP1 or other transcription factors, double immunofluorescent staining can be carried out using antibodies specific for AP1 and DIBALO in keratinocyte cultures derived from terminal and vellus hair bearing skin. Also ChIP-Seq analysis can be carried out in keratinocytes and fibroblasts where DIABLO has been silenced using siRNA to see if any gene expressions are affected such as IL1, EGF, PDGF, VEGF and TGFβ, which are involved and important in wound healing.

To this date, DIABLO has not been associated with other functions other than IAP and caspase interaction.

Xaf1 expression was high in both the epidermis and dermis of the terminal hair bearing skin and moderate in vellus hair bearing skin (Figure 2.9 and Table 2.8). The difference in expression could be due to vellus hear bearing skin being thinner than corresponding terminal hair bearing skin (Figure 2.7) (Vogt et al., 2007).

Its expression in the epidermis and dermis was higher compared to XIAP expression levels (Figure 2.8 and Table 2.7), indicating that Xaf1 may be regulating XIAP expression levels (Kempkensteffen et al., 2009).

Interestingly, Xaf1 was expressed at higher levels in the vellus hair follicle compared to the terminal hair follicle. It is not clear at which stage of anagen the hair follicles are at (Ebling, 1987), therefore expression of Xaf1 could potentially be due to the stage of the hair cycle, if in late anagen, apoptosis is about to occur for the catagen stage. Therefore expression could increase to prevent apoptosis from being inhibited. This is further evident, since in vellus hair follicles, Xaf1 expression was high whereas XIAP expression was low.

2.5 Conclusion

Immunofluorescent staining confirmed that in mice, the IAPs cIAP1, cIAP2, XIAP and Apollon and their antagonists DIABLO and Xaf1, were present in the skin and hair follicle while in humans, NAIP was also expressed. However, immunofluorescent staining is only semi-quantitative, further studies need to be conducted to quantitate the changes in the expression of IAPs and their antagonists in the hair cycle, particularly during extensive tissue remodelling.

In mouse skin, the expression of IAPs varied between different stages of the hair follicle cycle, indicating that IAPs potentially play a role in regulating the hair cycle.

Since anagen was induced by depilation, changes in the expression may also suggest a role in regulating apoptosis and maintaining cell homeostasis in a wound healing response.

In human skin, the difference in IAP expression between vellus and terminal hair follicles could be due to the stage in which the hair follicles are at, but could also be influenced by the type of hair follicle (vellus or terminal). The difference in dermal expression may be a reflection of the density of hair follicles, since vellus hair bearing skin contains smaller and less hair follicles compared to terminal hair bearing skin.

Ethically, it would be difficult to induce anagen via depilation in human skin to study IAPs and their antagonists in tissue regeneration and remodelling. Therefore, to study the roles of IAPs in human skin cells, the isolation and culture of primary cells is warranted.

Chapter 3

A Comparison of Cultured Human Dermal Fibroblasts Derived from Terminal and Vellus Hair Bearing Skin

3 A comparison of cultured human dermal fibroblasts derived from terminal and vellus hair bearing skin

3.1 Introduction

3.1.1 Dermal fibroblasts

Dermal fibroblasts are derived from the mesoderm (McAnulty, 2007) and are the major cell type in the dermis of the skin with an important role in constructing the ECM (Alberts, 2002). Fibroblasts are responsible for the synthesis of collagens, glycosaminoglycans, reticular and elastic fibres, and glycoproteins, which are all important components of the ECM. Dermal fibroblasts derived from different origins such as keloid scars, palmar, non palmar and plantar skin do not show the same plasticity, even when cultured under the same culture conditions (Chipev and Simon, 2002).

Fibroblasts from the same region, but from different layers of the dermis (reticular and papillary) also have different properties. Fibroblasts found in the upper papillary region of the dermis divide faster than fibroblasts cultured from the lower reticular layer of the dermis (Sorrell and Caplan, 2004). Different markers (Table 3.1) have been identified that distinguish between the two different fibroblast populations derived from the papillary and reticular layer (Janson et al., 2013). With aging, the number of fibroblasts present in the papillary layer is reduced, this therefore lowers the production of collagen and fibrous tissue in the ECM, reducing skin elasticity due to a reduction in elastic fibres (Janson et al., 2013; Montagna and Carlisle, 1979). A study by Mine et al. (2008) demonstrated that the dermal fibroblasts derived from the papillary layer of the dermis showed up regulation of the secretion of cytokines such as KGF, and VEGF in aged skin, with no change in expression with fibroblasts derived from the reticular layer.

Janson et al. (2013) compared the structure and characteristics of low and high passage fibroblasts derived from the papillary layer with matching patient low passage reticular fibroblasts and observed that specific reticular and

papillary markers (Table 3.1) were altered. Papillary marker expression was reduced in high passage (p15-20) papillary fibroblasts to similar levels seen in low passage (p3) reticular fibroblasts; while the expression of reticular markers was increased in high passage papillary fibroblasts. They concluded that papillary fibroblasts transformed into reticular fibroblasts after a high number of passages in culture.

Therefore, since cultured papillary fibroblasts at high passage differentiate into reticular fibroblasts, studies of papillary fibroblasts should be carried out at a low passage number to ensure the papillary fibroblasts retain their phenotype.

Papillary Markers:	Reticular Markers:
Podoplanin (PDPN)	Calponin1 (CNN1)
Netrin1 (NTN1)	Transglutaminase2 (TGM2)
C-C chemokine receptor type 11 (CCRL1)	Cadherin2 (CDH2)

Table 3.1: Papillary and reticular markers

Papillary and reticular fibroblast markers used to differentiate between the two layers (Janson et al., 2013)

3.1.2 Dermal fibroblasts in wound healing

The dermal fibroblast has an important role in human skin during wound healing (Werner et al., 2007). The existence of paracrine secretions between dermal fibroblasts and epidermal keratinocytes is well established (Nakajima et al., 2012; Tandara et al., 2007; Taniguchi et al., 2014). For example, keratinocyte communication with fibroblasts via interleukin 1 (IL1) secretion stimulates fibroblasts to secrete growth factors such as KGF, IL6 and granulocyte-macrophage colony-stimulating factor (GM-CSF). These are all important in wound healing, and have a positive feedback effect on keratinocytes to stimulate epidermal cell proliferation, while inducing fibroblasts to secrete collagen (Werner et al., 2007). The secretion of KGF from dermal fibroblasts also stimulates the proliferation of keratinocytes in the wound area (Sorrell and Caplan, 2004). The main role of the dermal fibroblast in wound healing is in the proliferative stage where they produce granular

tissue to support re-epithelialisation and rebuild the dermis. Dermal fibroblasts also have an important role in wound contraction, by differentiating into myofibroblasts, which are characterised by their high expression of α-SMA (Alberts, 2002; Hinz, 2007).

An *in vitro* study by Moulin et al. (2000) using a skin equivalent model, compared the effect of human myofibroblasts and dermal fibroblasts on keratinocyte re-epithelialisation. Wound healing myofibroblasts did not interact with keratinocytes to support keratinocyte differentiation and basement membrane formation in comparison to undifferentiated dermal fibroblasts (Moulin et al., 2000). In another *in vitro* study, a comparison of site matched papillary and reticular fibroblasts from breast skin, showed that the secretion of KGF1 by reticular fibroblasts was higher than corresponding papillary fibroblasts, suggesting that reticular fibroblasts may play a more dominant role in keratinocyte differentiation (Sorrell et al., 2004).

Another important aspect of wound healing is cell migration; fibroblasts need to migrate in wounded conditions to aid in repair efficiently. This is generally at the initial stage of wound healing, after platelets have cause clotting, forming a scaffold for fibroblasts to migrate (McDougall et al., 2006; Scheid et al., 2000).

A supply of stem cells ready to differentiate to replace damaged epithelial cells is important in wound healing. This generally involves the stem cells located in the skin epidermis, and in some cases where hair follicles are present, the stem cells in the bulge of the hair follicle (Ito and Cotsarelis, 2008; Ito et al., 2005; Plikus et al., 2012). It has been shown in mice that without the bulge stem cell niche, the skin is capable of wound repair, however, this niche allows for a faster rate of epithelialisation (Ito et al., 2005). The contribution of bulge stem cells to epidermal regeneration is only wound responsive. Wound healing is improved in skin where the hair follicle is in the anagen phase of the hair cycle (Ansell et al., 2011).

It has been established that the hair follicle mesenchyme fibroblasts, the dermal sheath, improve wound healing responses (Jahoda and Reynolds, 2001). During wound healing, where the outer root sheath contains the stem cells that contribute to epidermal regeneration, the dermal sheath plays a similar role for the contribution of dermal fibroblasts to the dermis. Myofibroblasts are derived from dermal fibroblasts, however, the dermal sheath cells already express high levels of α-SMA (Jahoda et al., 1991).

While the hair follicle clearly plays an important role in wound healing by contributing dermal sheath cells to the dermis (Jahoda and Reynolds, 2001), the role of the dermal fibroblasts contributing to the maintenance of the hair follicle is not clear. A study by Elliott et al. (1999) compared hair follicle dermal papilla size and cortex from male and female facial skin (male beard, female vellus hair) and scalp. Dermal papilla size in female vellus hair follicles was significantly smaller than androgen dependant male beard hair follicles. The enlargement of the dermal papilla was due to an increase in the number of cells either from cell proliferation, or from cells migrating from the dermal sheath (Elliott et al., 1999). However, there are no studies that have looked at the link between the number of fibroblasts in the region around vellus and terminal hair follicles, and whether that influences the type of hair follicle in the skin.

There are no studies comparing migration and proliferation, important characteristics of wound healing, between fibroblasts derived from hairy skin and non hairy skin in humans.

3.1.3 Aim

The previous chapter demonstrated that the expression of cIAP2, XIAP and NAIP was higher in terminal hair bearing skin compared to corresponding vellus hair bearing skin from the same donor.

The first aim of this chapter was to culture dermal fibroblasts from the papillary layer of the dermis of hairy – DF(T) (scalp) and non hairy – DF(V) (facial) skin

from the same donor, and compare their morphology and wound healing properties such as migration and proliferation *in vitro* at early passage.

FACS analysis of papillary dermal fibroblasts cultured from vellus (DF(V)) and terminal (DF(T)) hair bearing skin was used to compare cell morphology, a scratch wound assay to assess any differences in the rate of migration, and cell counting experiments to determine whether there are any differences in the rate of proliferation between the two fibroblast populations.

The second aim was to establish whether there is a difference in the expression of the IAPs, XIAP, NAIP, cIAP2 and Apollon, and the IAP antagonists DIABLO and Xaf1, between dermal fibroblasts cultured from terminal hair bearing skin and vellus hair bearing skin, using immunocytochemistry and qRT-PCR.

To investigate further the role of IAPs and their antagonists in controlling apoptosis in a wound healing response, fibroblast monolayers were scratched to create a mechanical wound, and changes in the expression of both IAP mRNA and protein quantified by qRT-PCR, and immunocytochemistry were examined.

3.2 Material and Methods

3.2.1 Cell culture

A list of donor details (e.g. age, gender and type of cell cultured) for the experiments presented in this chapter are listed in Table 3.2.

Donor ID	Age	Gender	DF(T)	DF(V)
OK14 [3, 4]	57	Female	✓	✓
OK15 [3, 4]	36	Female	✓	✓
OK17 [1, 3]	53	Female	✓	✓
OK18 [1, 2, 3, 4]	56	Female	✓	✓
OK21 [2]	64	Female	✓	✓
OK22 [1]	53	Female	✓	✓
OK24 [2]	57	Female	✓	✓

Table 3.2: Details, including internal ID, age, gender and location of which cultured dermal fibroblasts were derived.

Donors used to culture matching dermal fibroblasts from terminal hair bearing skin DF(T), or from vellus hair bearing skin (DF(V). [1] - used to FACS analysis, [2] - used for cell migration, [3] - used for cell proliferation, [4] - used for qRT-PCR analysis.

3.2.1.1 Isolation for primary hair follicle cell culture

For dermal fibroblast cell culture growth medium, DMEM (Sigma), was supplemented with 10% FBS (GIBCO), 100Units/mL, 100µg/mL Penicillin/ Streptomycin (GIBCO), 2mM L-Glutamine (GIBCO) and 0.25µg/mL fungizone (GIBCO).

Skin samples were obtained from the scalp and face of healthy females with full consent and ethical approval after undergoing facelift surgery. Matching skin samples were obtained from seven female subjects and used for FACS analysis (n=3, age range 53-56); cell migration (n=3, age range 56-64); cell proliferation (n=4, age range 36-57) and for qRT-PCR analysis (n=3, age range 36-57) -Table 3.2. The tissue was placed in transporting medium

(Dulbecco's Modified Eagle Medium (DMEM) (Sigma), 10% Foetal Bovine Serum (FBS) (GIBCO), 500Units/mL / 500µg/mL Penicillin/ streptomycin (GIBCO), 0.75µg/mL fungizone (GIBCO), glutamax (GIBCO)) and stored at 4˚C for up to 12 hours. Each sample was divided into the terminal hair bearing scalp region and the vellus hair bearing facial skin (Figure 3.1). This allowed a direct comparison of dermal fibroblasts cultured from terminal hair bearing skin (DF(T)) and vellus hair bearing skin (DF(V)) of the same donor. Tissue for cell culture was used within 24 hours of surgery.

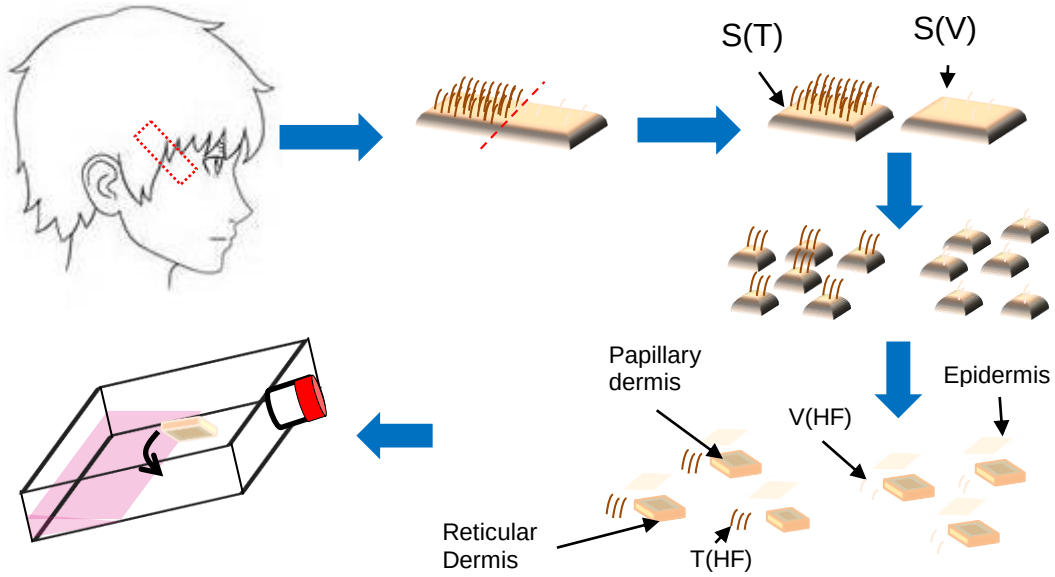


Figure 3.1: Terminal and vellus hair bearing skin separation for dermal fibroblast cell culture

Skin samples obtained from female face lift patients – marked red dashed box were isolated into two sections, terminal hair bearing and vellus hair bearing skin – dashed red line. Skin samples from both terminal and vellus hair bearing skin were then cut into smaller 1cm^2 pieces. Samples were placed into a 6cm diameter culture dish with medium, the epidermal layer was removed using a scalpel and scissors with minimal disruption of the dermis. For DF(V) cell culture, cells were placed papillary layer onto the surface of the T25 culture flask; for DF(T) cells, once the epidermis was removed, hair follicles were plucked and papillary layer of the dermis was placed onto the surface of the T25 culture flask. S(T) – terminal hair bearing skin, S(V) – vellus hair bearing skin, T(HF) – terminal hair follicle, V(HF) – vellus hair follicle.

Skin obtained from surgery was washed with sterile PBS. For dermal fibroblasts obtained from terminal hair bearing skin (DF(T)), excess fat was removed with a thin layer of dermis from the isolated skin section. The epidermis was removed using a sterile blade, leaving the papillary layer and reticular layer of the dermis. Hair shafts still protruding were removed using sterile tweezers. The dermis was divided into smaller pieces of roughly $1cm^2$. The isolated dermal tissue pieces were placed in a T25 culture flask that had already been coated with 500µl of FBS and left to air dry in the laminar flow cabinet, papillary layer face down onto the surface of the flask. The flasks were scored using the sterile tweezers and the individual small piece dermis were placed close to one another; 5-6 pieces were placed in one coated flask. The flasks were carefully topped up with 3ml growth medium so as not to dislodge the tissue pieces and were incubated at 37˚C and 5% CO_2 for 1 week without disturbing before the medium was replenished. After approximately 1-2 weeks fibroblasts had started to migrate out from the dermal explants in all directions (as observed under a phase contrast microscope). Once the cells were established (~4 weeks) and a confluent monolayer of primary dermal fibroblasts had grown, the cells were trypsinised, and seeded into a T75 culture flask for expansion.

Using the same principle, matching dermal fibroblasts from vellus hair bearing skin were cultured from the same biopsies; cells took approximately 3 weeks to become confluent before the first passage.

3.2.1.2 Passaging of cells

Once cells had reached confluence, they were passaged, and transferred to new culture flasks. In a T75 flask 1.5ml of 0.05% trypsin/EDTA (GIBCO) was added to the cells; for T25 flasks, 0.75ml of 0.05% trypsin/EDTA (GIBCO) was added. The fibroblasts were examined under a microscope; the cell morphology changed from slightly dendritic in shape to round cells. Once all the cells had shrunk (~1-2 min of adding trypsin), the trypsin solution was removed, and the cells incubated at 37˚C for a further 1-2 minutes. The flask

was tapped gently to detach the cells from the surface of the flask and growth medium was added to the flask and the cells re-suspended (in T75 flasks, 8mls of medium was added; in T25 flasks, 4mls of medium was added). From the cell suspension, a one in four dilution was carried out on the dermal fibroblasts; 2mls of cell suspension was transferred to a new T75 flask and topped up with 5ml growth medium; for T25 flasks, 1ml of cell suspension taken and transferred to new flask topped up with 2mls of growth medium.

3.2.1.3 Cell counting

Once the cells were confluent, the cells were trypsinised and 10µl of cell suspension was placed in an improved Neubauer haemocytometer. The number of cells in the corner and middle compartments were counted, and only two edges (bottom and left) of each grid were used. The average number of cells were taken and multiplied by 10^4 to accommodate for the volume of the haemocytometer ($0.1mm^3$); this gave the number of cells in 1 ml. Using this information, the number of cells in a confluent flask suspension could be calculated.

3.2.1.4 Freezing cells

Once cells had reached confluence, and needed to be stored for future use, the cells were frozen. Cells were trypsinised and re-suspended in 10% FBS DMEM in a falcon tube and centrifuged at 560RPM for 5 minutes to form a pellet. The supernatant was discarded and the pellet was re-suspended in 2ml freezing media which consisted of 10% dimethyl sulfoxide (DMSO) in 20% FBS DMEM. The 2ml of cell suspension was divided equally between 2 labelled cryotubes. Cryotubes were placed in liquid nitrogen vapour for 1 day, and then placed into liquid nitrogen.

When cells were required, they were removed from the liquid nitrogen and immediately thawed in hot water. One vial of cells was transferred into a T75 culture flask and topped up with 9ml 10% FBS DMEM. The cells were left to incubate at 37˚C with 5% CO_2, with the medium changed after 24hours.

3.2.2 FACS analysis for cell morphology

DF(T) and DF(V) cells were cultured in T75 flasks and left to grow until fully confluent. A cell suspension was made with 300,000 dermal fibroblasts in 300µl PBS. Cells in each flask were counted and from the total number of cells, 300,000 cells were extracted and placed into an Eppendorf tube and spun down at 3000g for 4 minutes, before the pellet was made up to 300µl using PBS. The cell suspension was transferred into a standard FACS tube and placed into a FACS machine (Beckman Coulter). Using the specific software (Summit v.4.3.02.) a spot area was created with the x-axis a forward linear scatter, where any light scatter that is in the direction of light beam is collected, this indicates the size of the cells analysed; this is against the y-axis which is the side scatter, also linear range. The light scattered from the cell that is in any other direction but towards the beam is collected. This provides information on cell complexity, including the amount of cytoplasmic granulation and nuclear size. The voltage and gain for the side scatter is adjusted so cells appear in the plot area; the gain for the forward scatter is also adjusted. The threshold is adjusted to remove any dead cells and debris that could affect analysis. A gate is placed around the cell population that needs to be analysed. The samples were run and cells were analysed in terms of size (x-axis) and complexity (y-axis). This was carried out for three sets of matching dermal fibroblast cell cultures.

3.2.3 Assessment of cell migration using the scratch wound assay

Matching cultures of dermal fibroblasts (DF(T) and DF(V) cells) were seeded at a concentration of 200,000 cells per well in a 6 well plate (Clear Sterile) and left to grow to fully confluent (DF(V) – 4 days, DF(T) – 1 week). The medium was changed every 3 days and once cells had reached confluence, they were washed with PBS twice, then incubated in serum free, phenol red free DMEM for 24 hours.

Using the scratch wound device (Figure 3.2A), a scratch with a diameter of 0.8mm was made down the middle of the well. The cells were washed twice with PBS to remove any dead or detached cells. 2ml of serum free and phenol red free medium was added to the cells. To measure only migration and not proliferation, cells were treated in the presence and absence of mitomycin C (5µg/ml and 10µg/ml), to ensure cell proliferation was inhibited.

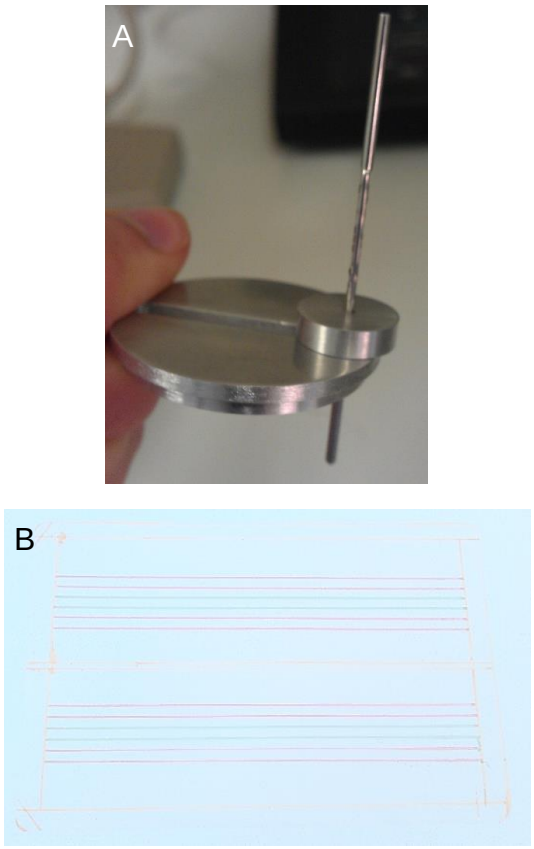


Figure 3.2: Scratch wound device and template

A) This device was used to scratch wounds in 6 well plates to give an even thickness scratch without damage to the plates and to give a neat straight scratch across the cells. The rim sits onto the 6 well plate, and the bar is 0.8mm in diameter at the base. B) Template used to indicate migration points. This template was placed under the 6 well plate in which cell migration was measured and secured using tape. Colours were used for reference points.

3.2.3.1 Analysing cell migration

Once the cell monolayers were scratched, a template (Figure 3.2B) was placed under the plate. Using a camera (Nikon coolpix4500 – 4x lens magnification, Japan) attached to the phase contrast microscope (Ernst Leitz Wetzlar GMBH, Germany); images of the scratched cells were taken at x4 objective magnification (between the two green lines) and x10 objective magnification at each template line.

Using a stage micrometer, images were taken of the 1mm length at both objective magnifications, this was to convert pixel length to actual length for migration analysis.

Migration was analysed at different time points, at 0, 2, 4, 6, 24 and 48 hours after scratching to analyse at which time point dermal fibroblast migration was affected under different experimental conditions.

3.2.4 Quantitating DF(T) and DF(V) cell proliferation

Four matching donor DF(T) and DF(V) fibroblast cultures were seeded into 6 well plates at 10,000 cells per well. For each individual donor cell type, three 6 well plates were set up for each experimental condition. Cells were incubated with 5% charcoal stripped FBS in phenol red free medium. Cells were counted at specific time points for 12 days, at 2, 4, 6, 8, 10 and 12 days of incubation, medium was changed every 2 days. Dermal fibroblasts were cultured in triplicate wells and cells were counted twice in each individual well.

3.2.5 Quantitative PCR

To analyse the expression of different RNA transcripts and how they were affected under different experimental conditions, quantitative PCR was carried out.

3.2.5.1 RNA extraction

DF(T) and DF(V) cells were seeded at 20,000 cells per well in a 6 well plate (each experimental condition was done in triplicate wells) and allowed to grow

until fully confluent (DF(V) – 1 week, DF(T) – 2 weeks) with growth medium change every three days. Once cells were fully confluent, medium was changed to serum free and phenol red free medium and cells were incubated for 24hours. Cells were either scratched or left unwounded, and treated with vehicle control (absolute ethanol at 0.001%). Mechanical wounding was achieved by using a 1ml pipette tip and scoring a hash (#) pattern in the wells. The cells were washed with PBS to remove any dead or detached cells before treatment.

After a 24hour incubation, cells were trypsinised and collected in 20ml falcon tubes by washing the wells containing trypsinised cells with serum free and phenol red free medium; cells from triplicate wells were pooled together, then centrifuged at 560RPM for 5 minutes. The RNeasy Mini kit (Qiagen) was used for RNA extraction. Cells were lysed using a Buffer RLT (Qiagen kit) solution (350µl), then 70% ethanol (350µl) was added to the lysate for binding purposes. 700µl of the homogenised cells were transferred to spin columns (provided in the kit), and spun down at 8000g for 15 seconds. The flow through was discarded and 500µl of Buffer RPE (Qiagen kit) was added to the spin column and spun at 8,000g for 15 seconds; the flow through was discarded and this step was repeated twice more, however the solution was spun for 2 minutes each time. The spin column was placed in a new collecting tube and 40µl of RNase free water (Qiagen kit) was added to the spin column and spun at 8,000g for 1 minute to elute the RNA. The RNA was aliquoted in volumes of 10µl in 200µl Eppendorf tubes to reduce damage to RNA by constant freeze-thawing (~35 µl of RNA was collected from the triplicate wells).

3.2.5.2 cDNA synthesis

Once the RNA had been extracted, the quantity of RNA in ng/µl was measured using a NanoPhotometer P-330 (Implen). Reverse transcription was carried out to create cDNA, using the ImProm-II™ Reverse Transcription System kit (Promega) and protocol. 1µg of RNA was added to 1 µl of random primer (Promega kit) and topped up to 5µl with DNase/RNase-Free Distilled Water

(Promega kit). This solution was placed on a preheated 70˚C thermocycler (Techne) for 5 minutes. Immediately, the PCR tubes were placed on ice for at least 5 minutes, then to collect all the condensate the tubes were spun for 10 seconds on a small refrigerated centrifuge (Eppendorf 5415 R). To each reaction 4µl of reaction buffer, 2.5µl of magnesium chloride, 1µl of dNTP mix, 0.5µl of recombinant RNasin Ribonuclease Inhibitor and 1µl of reverse transcriptase enzyme, nuclease free water was added to make up to a 15µl solution (all reaction mixture provided from Promega reverse transcription kit); this was then added to the 5µl RNA solution making a total of 20µl solution. The solution in the PCR tubes were placed in a thermocycler (Techne) programmed to heat to 25˚C for 5 minutes for annealing purposes; then to increase in temperature to 42˚C for one hour for extension. The reaction mix was inactivated by increasing the temperature to 70˚C for 5 minutes. The cDNA created was stored at -80˚C.

3.2.5.3 Quantitative real time PCR analysis

Primer sequences were designed for the IAPs, Apollon, cIAP2, NAIP, XIAP and the IAP antagonists DIABLO and Xaf1

Primer:	Forward Sequence:	Reverse Sequence:	Annealing Temp:
Apollon	CAGCCAACCTTACCTCTCCG	GTCCGCAGTCCTACCAACAA	62.2˚C
cIAP2	GGGCTGTTACCGCTGAGAAT	ACGTTGGGCTTTTCAACTGC	62.2˚C
NAIP	AGCTGGAAGTGGAAAGACGG	GCTGGATAATGTTCCTCACGC	58.6˚C
XIAP	AGCAATTGGCTTTTTCCCACT	CAAGAAAGGTAGCTTGGTGAAGT	62.2˚C
DIABLO	CCAATCCCGACTGCTTCCTT	ACAGCCAACTCTTCAGAGCC	62.2˚C
Xaf1	TGGGTGTAGGATTCTCCAGG	GGTTTGCCCAAGGACTACAA	62.2˚C
GAPDH	TATAAATTGAGCCCGCAGCC	CGACCAAATCCGTTGACTCC	62.2 and 58.6˚C

Table 3.3: IAP primers used and their annealing temperature

Each primer was designed using PrimerBlast and annealing temperature optimised via quantitative PCR optimisation

3.2.5.3.1 Primer annealing temperature optimisation

The designed primers (Table 3.3) were optimised for their annealing temperature. For each reaction, 5µl of Fast SYBR® Green Master Mix (life

Technologies), 0.1µl of forward and 0.1µl of reverse primer, 0.3µl of cDNA and 4.5µl of UltraPure™ DNase/RNase-Free Distilled Water (GIBCO) were mixed together, and analysed at different temperatures ranging from 55˚C to 65˚C. The mixture was placed in a 96 well PCR plate (Starlab) and sealed using clear flat caps (Starlab), the mixture was quickly vortexed then spun down using a plate spinner (Labnet MPS1000) to collect the solution at the bottom of the well ensuring no bubbles were present. The plate was placed into a quantitative real time PCR machine (Step One Plus, Applied Biosystems) and a designed protocol was run. The protocol used was to heat to 95˚C for 20 seconds for initial activation, the first cycle began after the temperature was maintained at 95˚C for 3 seconds, then reduced to the temperature ranges for optimisation for 30 seconds. These two steps were repeated for the number of cycles that were required (40 cycles). A melt curve was produced to determine whether the product had amplified. This stage consisted of the temperature after the final cycle to increase to 95˚C for 15 seconds, then drop to 65˚C for 1 minute then increase back to 95˚C with the reading on the product at every increase of 1˚C.

3.2.5.3.2 Real time PCR

Once the optimal annealing temperature was established, using the same protocol for establishing the optimal annealing temperature for each individual primer, the experiment was repeated with the modification of the actual annealing temperature (Table 3.3) as opposed to the temperature ranges. For each condition and primer analysis, the reaction was done in triplicate wells. For each experimental condition, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) a catalyst enzyme in glycolysis used to break down glucose for energy, was used as a house keeping gene where levels were unaltered at different conditions. Once the PCR had run, a CT value was generated for the reaction. From the CT values generated, a ΔCT value was generated from the house keeping gene (the expression of the gene of interest normalised against GAPDH – the change in CT value in comparison to GAPDH); from that a ΔΔCT was further generated to ensure that the expression could be

obtained. For consistent purposes, expression was normalised against GAPDH.

3.2.6 Expression of IAPs in dermal fibroblast monolayer culture at different wounding conditions via immunofluorescent staining

Matching cultures of DF(T) and DF(V) cells from three different donors were seeded into 8 well chamber slides (Millipore) at a density of 3,000 cells per well and left to grow until fully confluent (DF(V) – 4 days, DF(T) – 6 days). Growth medium was changed every three days. When cells became fully confluent, one well for each IAP and IAP antagonist analysed was scratched at one side of the well and left for 24 hours. After 24 hours, a scratch in another well was made using a 20µl pipette tip. Immediately after scratching, medium was removed and cells were left to air dry before fixed using ethanol. Using the same principle of immunofluorescent staining described in section 2.2.4, the expression of IAPs and their antagonist was determined in matching DF(T) and DF(V) cells non wounded, immediately after wounding (T_0) and 24 hours later (T_{24}). Two random images were taken from each well. Once the images were taken, using ImageJ, six random cells were analysed, where their fluorescent density was measured (Figure 3.3).

3.2.7 Statistics

For all FACS analysis, cells migration assay between two groups and PCR data carried out, an un-paired Students T-test of unequal variance was conducted, and a one way analysis of variance (ANOVA) on multiple comparisons for the same protein for immunofluorescent quantitative analysis followed by a Bonferroni test, to determine significance between specific groups.

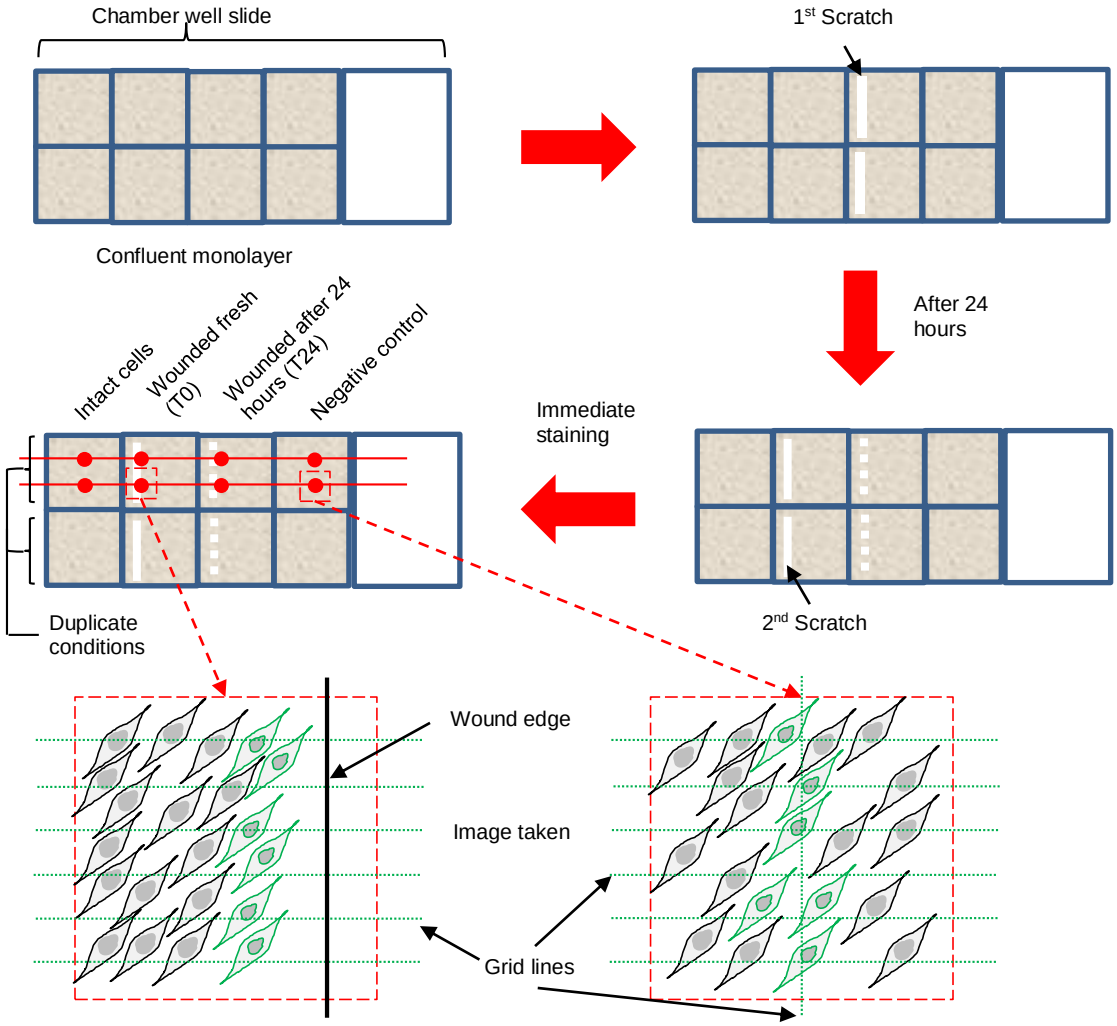


Figure 3.3: Schematic representation of wounding method of confluent monolayer in chamber slides and image positions

Confluent DF(T) ad DF(V) monolayers were scratched (white line) at the edge of the chamber (this will create the wounding condition after 24 hours). Cells were incubated for 24 hours, and a second scratch was made in another well (this will create the wounding condition at time zero). Cells were immediately fixed and immunostaining carried out in duplicate. Random images were taken (marked with a red dot). Each image was analysed using ImageJ, from each image, six cells were analysed (green outline). Unwounded cells, those at the point of intersection on the template gridline were analysed, where cells were wounded, those at the edge of the wound were analysed.

3.3 Results

3.3.1 DF(V) are smaller and more complex than DF(T)

To quantitate any differences between DF(T) and DF(V) cells, FACS analysis was used to determine the size and cell complexity of the two different dermal fibroblast populations (Figure 3.4). From the dot-plot graph generated, the size and complexity of the cells were compared. Figure 3.4 represents the cell morphology for DF(T) and DF(V) cells. Dermal fibroblasts from three matching donors were analysed, generating data for the cell size (x-axis) and the cell complexity (y-axis). When normalising the data against DF(T) cells, DF(V) cells are smaller than DF(T) cells ($p<0.05$), and DF(V) cells are more complex in cytoplasm granulation and nuclear size compared to DF(T) cells ($p<0.05$), (Student T-test).

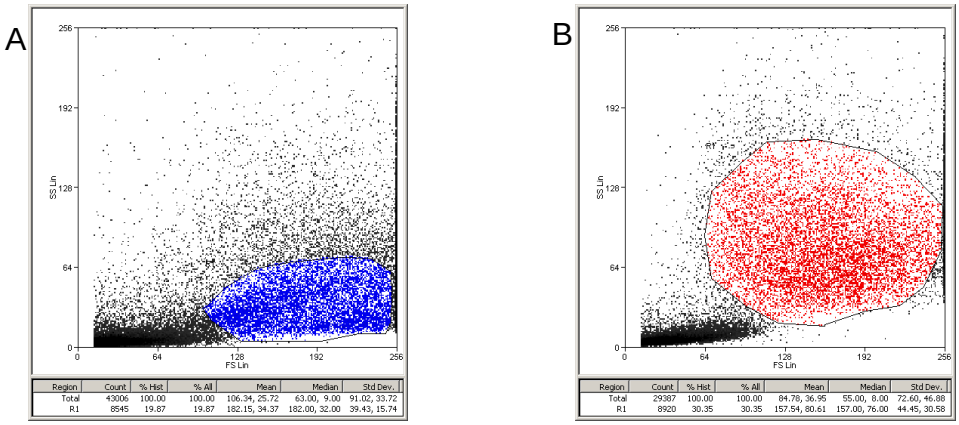


Figure 3.4: Differences in DF(T) and DF(V) cell morphology under FACS analysis

Cell morphology for DF(T) and DF(V) under FACS analysis. A) DF(T), B) DF(V). DF(V) were smaller than DF(T) cells from matching donors. DF(V) were more complex in cytoplasm granulation. Blue – DF(T), red – DF(V). n=3 donors.

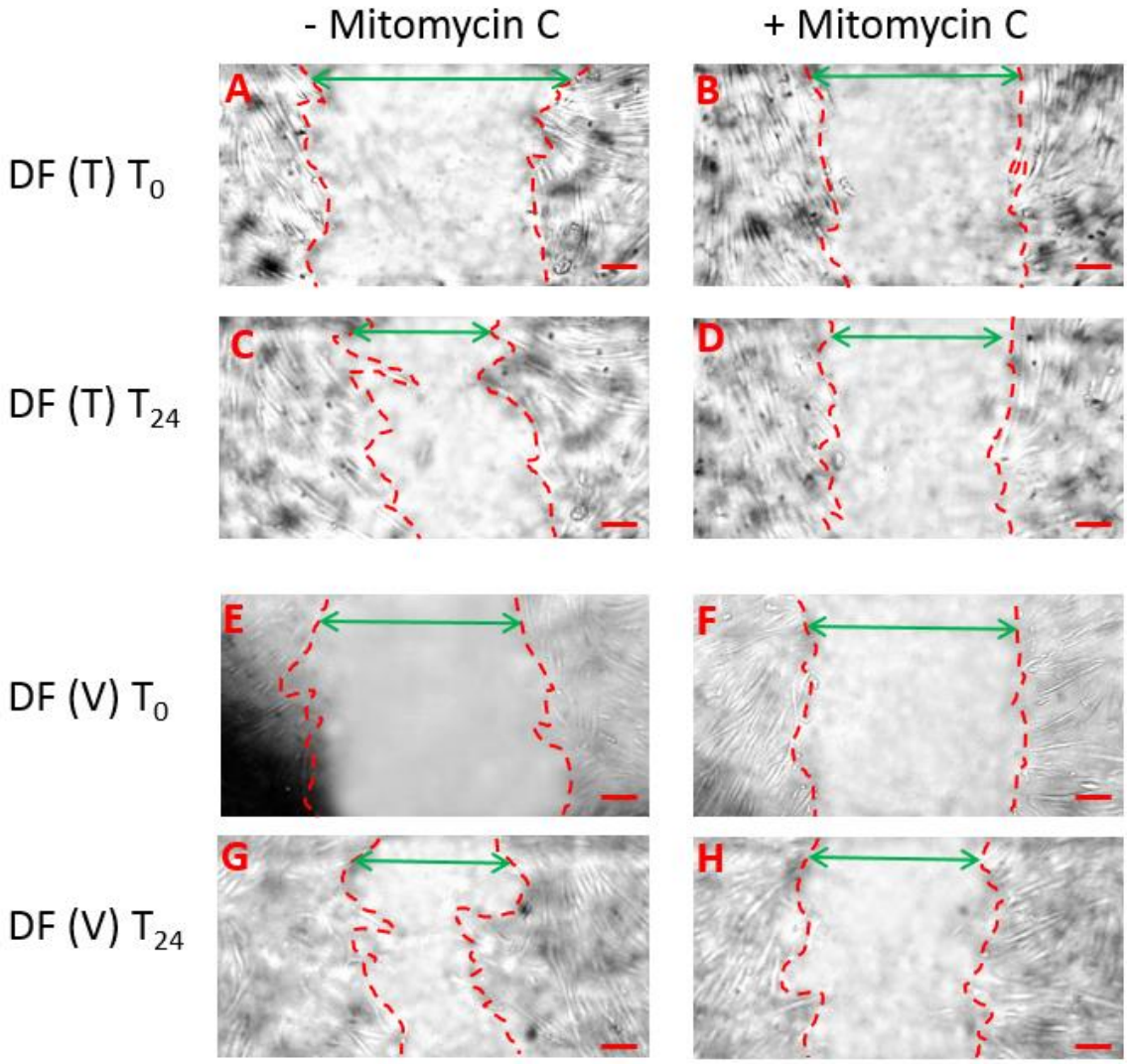
3.3.2 A comparison of the migratory ability of dermal fibroblasts derived from terminal and vellus hair bearing skin

A cell migration assay was carried out on matching cultures of dermal fibroblasts (n=3 donors) 24 hours after scratching; all cells were analysed at passage 3 (Figure 3.5).

Figure 3.5A and Figure 3.5B, represent DF(T) cells immediately after scratching in the absence and presence of 10µg/ml mitomycin C respectively. Figure 3.5C and Figure 3.5D represent DF(T) cell migration 24 hours following scratching in the absence and presence of 10µg/ml mitomycin C respectively. Cell migration was reduced with treatment of cells with mitomycin C.

Figure 3.5E and Figure 3.5F, represent DF(V) cells immediately after scratching in the absence and presence of 10µg/ml mitomycin C respectively. Figure 3.5G and Figure 3.5H represent DF(V) cell migration 24 hours following scratching in the absence and presence of 10µg/ml mitomycin C respectively. As with DF(T) cells, DF(V) cell migration was reduced with mitomycin C treatment.

With mitomycin C treatment, cell migration was reduced 24 hours after scratching in both DF(T) and DF(V) cells, $p<0.005$ (Figure 3.5I). No difference in cell migration was observed between DF(T) and DF(V) cells in the presence or absence of 10µg/ml mitomycin C.


- Mitomycin C
+ Mitomycin C
DF (T) T_0
DF (T) T_{24}
DF (V) T_0
DF (V) T_{24}
A
B
C
D
E
F
G
H

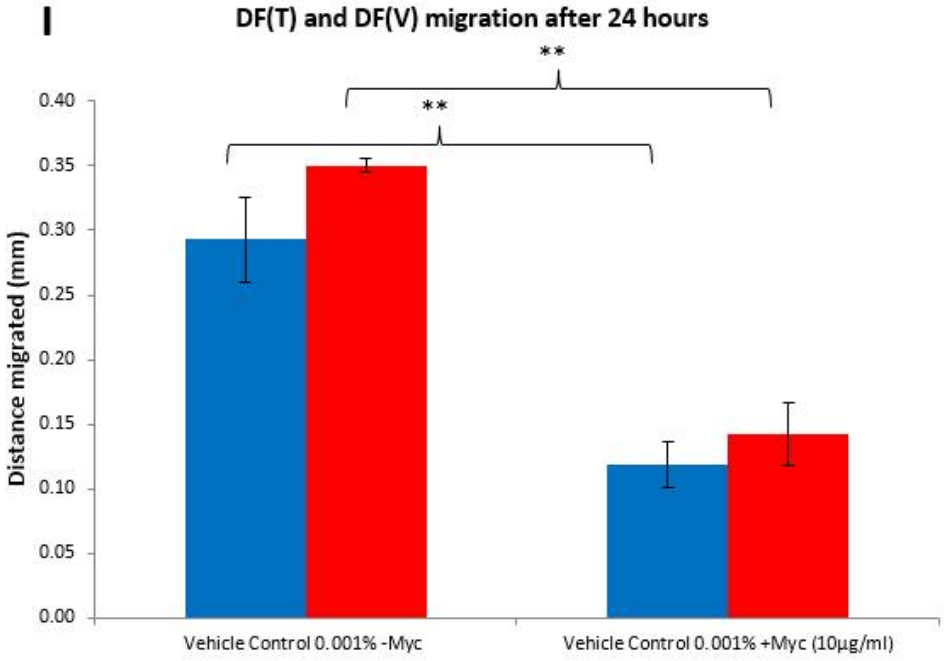

I
DF(T) and DF(V) migration after 24 hours
**
**
Distance migrated (mm)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Vehicle Control 0.001% -Myc
Vehicle Control 0.001% +Myc (10µg/ml)

Figure 3.5: Dermal fibroblast migration DF(T) and DF(V) in the presence and absence of Mitomycin C

*The migration of dermal fibroblasts in a scratch wound assay after 24 hours. A and B) Immediate scratching of DF(T) cells in the absence and presence of 10µg/ml mitomycin C respectively. C and D) 24 hours after scratching of DF(T) cells in the absence and presence of 10µg/ml mitomycin C respectively. E and F) Immediate scratching of DF(V) cells in the absence and presence of 10µg/ml mitomycin C respectively. G and H) 24 hours after scratching of DF(V) cells in the absence and presence of 10µg/ml mitomycin C respectively. I) Comparison of the migration of DF(T) (blue) and DF(V) (red) cells 24 hours after scratching, in the presence and absence of Mitomycin C. n=3, T-Test, ** p<0.005. Magnification bar 100µm.*

3.3.3 DF(V) proliferate at a faster rate than DF(T)

To determine whether there were differences between the proliferation of dermal fibroblast populations derived from terminal or vellus hair bearing skin, matching cultures were established from four different donors; fibroblasts were all assayed between passage 3-4 (Figure 3.6). There were significant differences in proliferation between the two dermal fibroblast populations over a 12 day period. By day two, DF(V) cells were proliferating significantly faster than DF(T), which was maintained throughout the 12 day period.

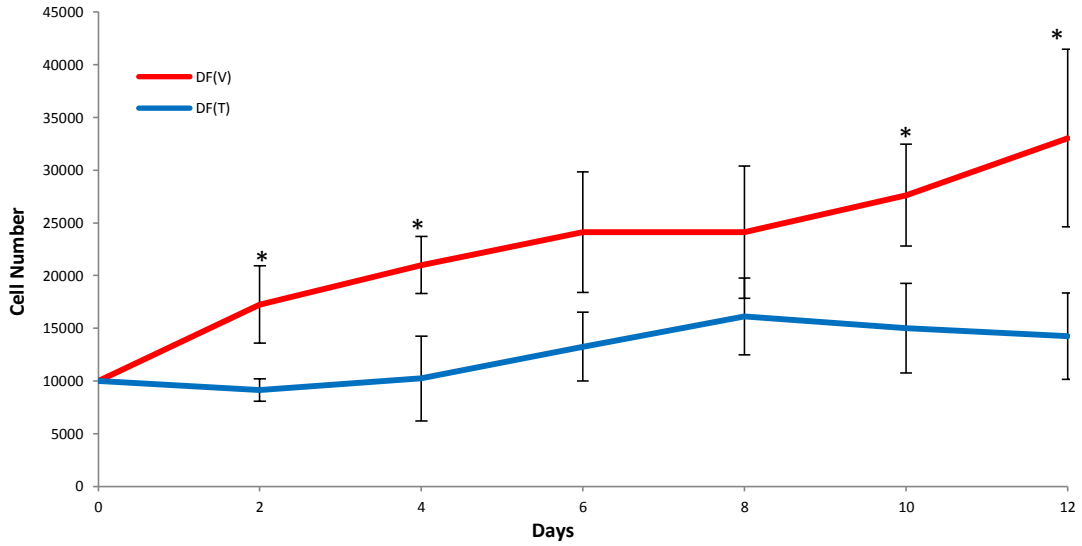


Figure 3.6: DF(V) grow significantly faster than DF(T)

*Proliferation of matching cultures of DF(T) (Blue) and DF(V) (Red) derived from the same donor (n=4 donors) passage (3-4). Each cell line for each individual patient was assessed in triplicate wells for each time point and each well was counted in duplicate. There was a significantly increased rate of proliferation by DF(V) compared to DF(T) over a 12 day period. Each point represents mean of 4 donors ± SEM. * p<0.05 (Student t-test).*

3.3.4 DF(V) express higher levels of NAIP, cIAP2, Apollon and DIABLO mRNA than DF(T)

Quantitative RT-PCR was carried out to compare mRNA expression for IAPs and IAP antagonists in matching DF(T) and DF(V) cells from three donors (Figure 3.7). All expressions were normalised against GAPDH.

Messenger RNA expression for NAIP was ~5x higher in DF(V) cells compared to matching DF(T) cells ($p<0.0005$), while cIAP2 mRNA expression was ~20x higher in DF(V) cells compared to corresponding DF(T) cells ($p<0.0005$). mRNA expression for XIAP was similar, while expression of Apollon was ~2.7x higher in DF(V) cells compared to matching DF(T) cells ($p<0.05$) (Figure 3.7A).

While the expression of DIABLO was ~1.4x higher in DF(V) cells compared to matching DF(T) cells ($p<0.05$) (Figure 3.7B), there was no significant difference in the expression of Xaf1 (Figure 3.7C).

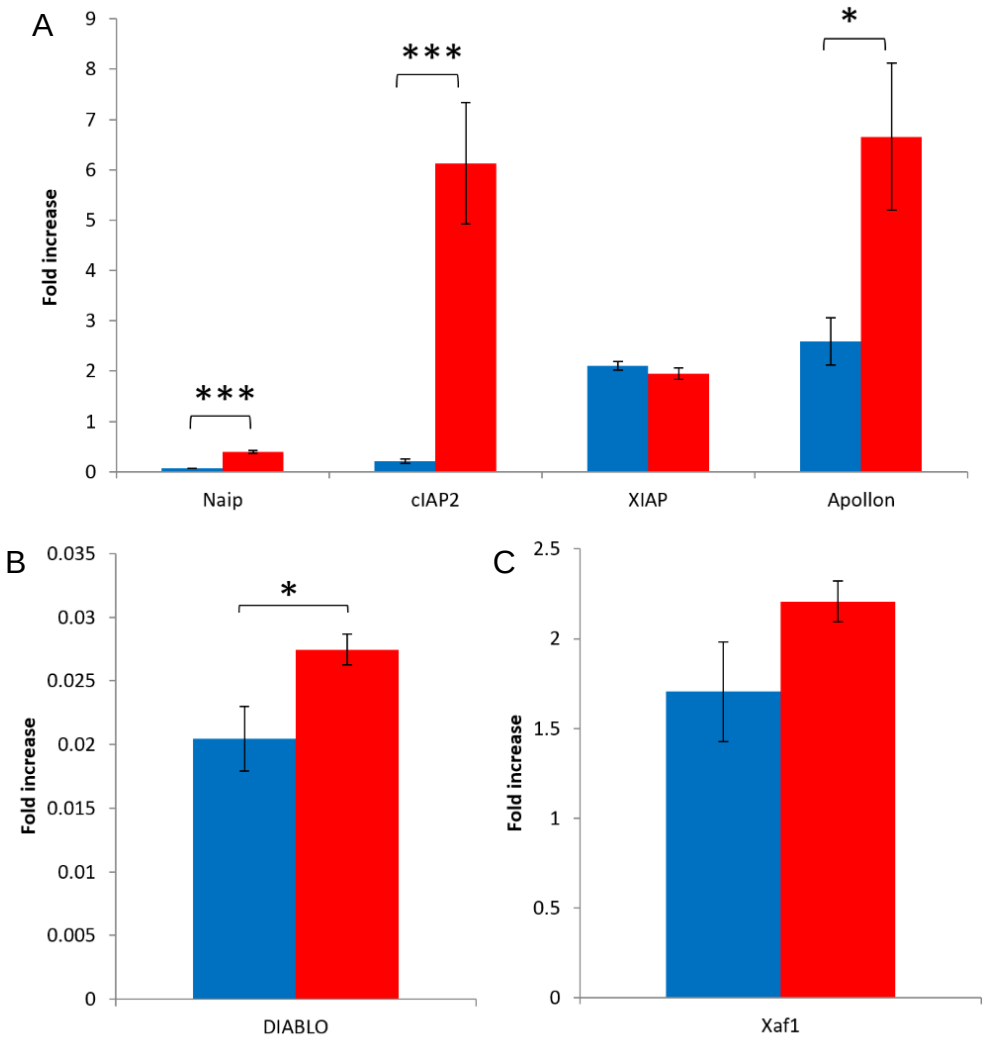


Figure 3.7: Higher levels of NAIP, cIAP2, Apollon and DIABLO in DF(V) cells compared to DF(T) cells under non-wounding conditions.

*A comparison of mRNA expression of IAPs and IAP antagonists between matched cultures of DF(T) (blue) and DF(V) (red). A) Expression of mRNA for NAIP, cIAP2, XIAP and Apollon. (B and C) Expression of mRNA for the IAP antagonists DIBALO and Xaf1 respectively. * p<0.05, *** p<0.0005, (Student T-test). n=3 donors in triplicate wells.*

3.3.5 Changes in mRNA expression of IAPs differ between DF(T) and DF(V) cultures following mechanical wounding

High levels of mRNA expression for XIAP and Apollon were seen in DF(T) compared to expression of NAIP and cIAP2; this pattern of expression was also seen in cells 24 hours following mechanical wounding. However, in DF(V) cells, expression of cIAP2 and Apollon was higher than XIAP and NAIP. 24 hours following mechanical wounding in DF(V) cells, Apollon and XIAP mRNA expression was higher than NAIP and cIAP2 (Figure 3.8).

In DF(T) cells (n=3 donors) (Figure 3.8A), mRNA expression of cIAP2 and XIAP was reduced 24 hours after the cultured cell monolayers were mechanically wounded ($p<0.05$); but there was no change in the expression of NAIP and Apollon mRNA. In the matching DF(V) cells (Figure 3.8B), cIAP2 mRNA expression was reduced by a sixth in mechanically wounded cells compared to unwounded ($p<0.0005$), whilst Apollon mRNA expression was decreased by a half following mechanical wounding ($p<0.05$).

In contrast to DF(T), where there was a decrease in XIAP mRNA expression 24 hours after scratching, there was an increase in the expression of XIAP in DF(V) cells ($p<0.05$).

Similar to DF(T), NAIP mRNA expression was not altered 24 hours after wounding in DF(V).

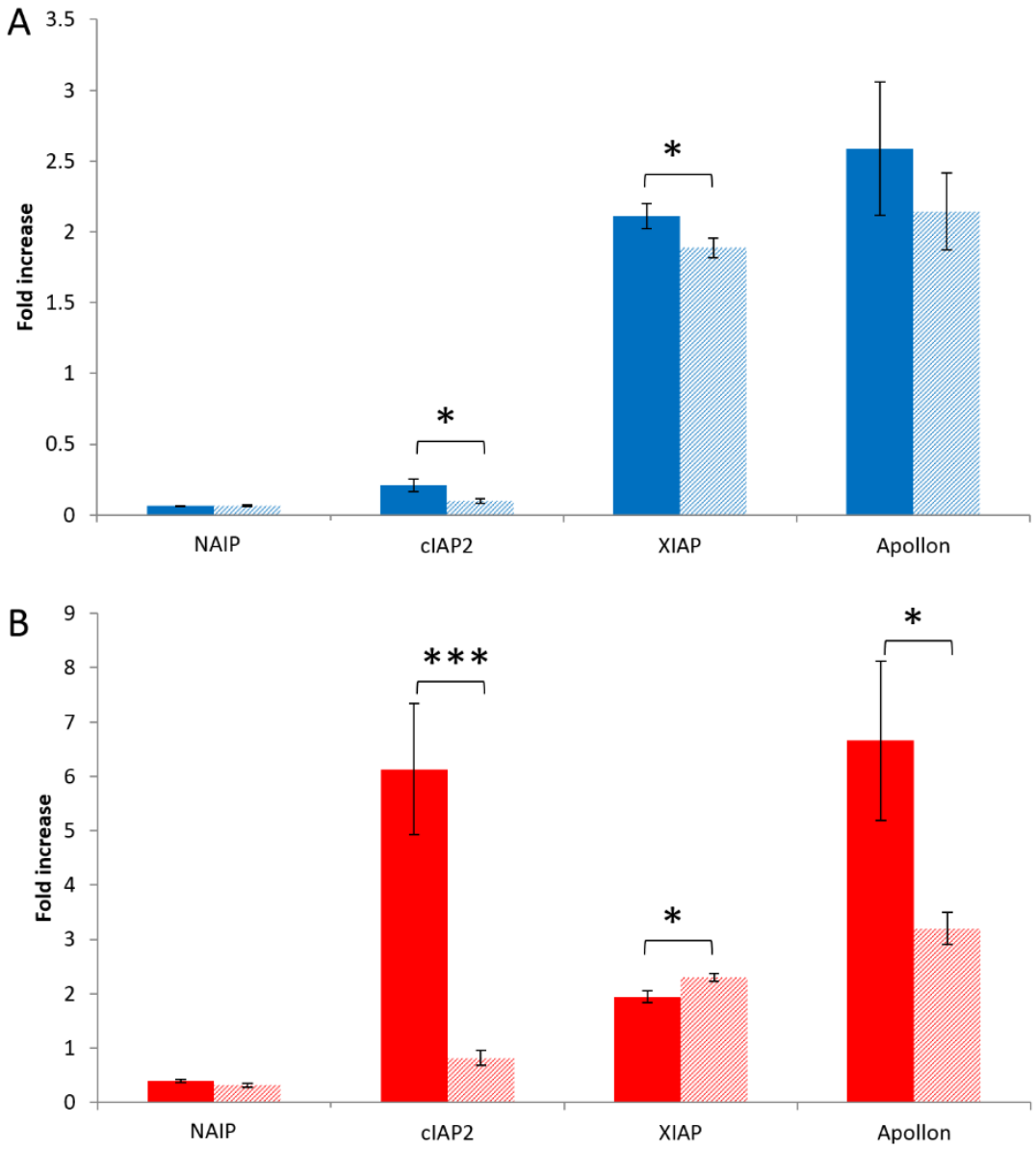


Figure 3.8: Decreased levels of cIAP2 and XIAP in DF(T) cells with decreased levels of cIAP2 and Apollon and increased levels of XIAP in DF(V) following mechanical wounding *in vitro*

*Confluent monolayers of dermal fibroblasts were mechanically wounded (scratched), and the level of IAP mRNA compared between matching DF(T) and DF(V) cultures. A) Represents IAP expression levels in DF(T) before (blue) and 24 hour after wounding (hashed blue). B) Represents IAP expression levels in DF(V) before (red) and 24 hour after wounding (hashed red). n=3 donors. * p<0.05, *** p<0.0005, (Student T-test).*

3.3.6 IAP antagonist mRNA expression decreases in cultured DF(T) and DF(V) after mechanical wounding

In matching DF(T) and DF(V) cultures, Xaf1 mRNA expression was higher compared to DIABLO expression, in unwounded and 24 hours following mechanical wounding (Figure 3.9).

mRNA expression for DIABLO reduced by a half in DF(T) cells following mechanical wounding ($p<0.005$) (Figure 3.9A), whilst in DF(V) cells DIABLO mRNA expression was reduced by a quarter after cells were mechanically wounded ($p<0.05$) (Figure 3.9C).

Xaf1 mRNA expression was not altered following mechanical wounding in DF(T) cells (Figure 3.9B), however, in DF(V) cells, expression was reduced by about 25% following mechanical wounding ($p<0.005$) (Figure 3.9D).

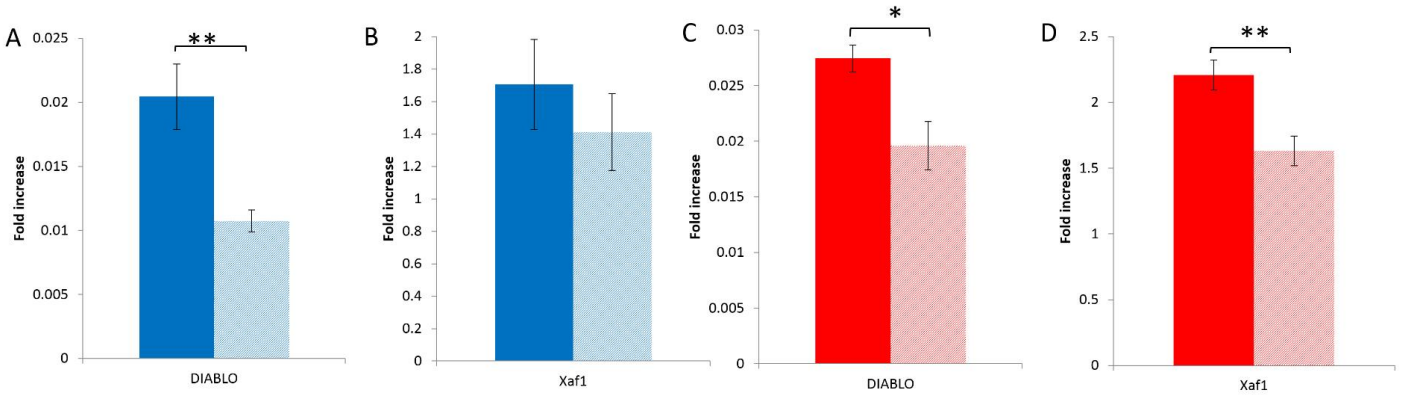


Figure 3.9: Significant decreases for DIABLO in DF(T) and significant decrease in DIABLO and Xaf1 in DF(V) cells following mechanical wounding *in vitro*

*Confluent monolayers of dermal fibroblasts were mechanically wounded (scratched), and the level of IAP antagonist mRNA compared between matching DF(T) and DF(V) cultures. (A and B) Represents DIABLO and Xaf1 mRNA expression levels respectively in DF(T) before (blue) and 24 hour after wounding (hashed blue). (C and D) Represents DIABLO and Xaf1 mRNA expression levels respectively in DF(V) before (red) and 24 hour after wounding (hashed red). n=3 donors. * p<0.05, ** p<0.005, (Student t-test).*

3.3.7 Protein expression of IAPs in unwounded and mechanically wounded DF(T) and DF(V) cultures

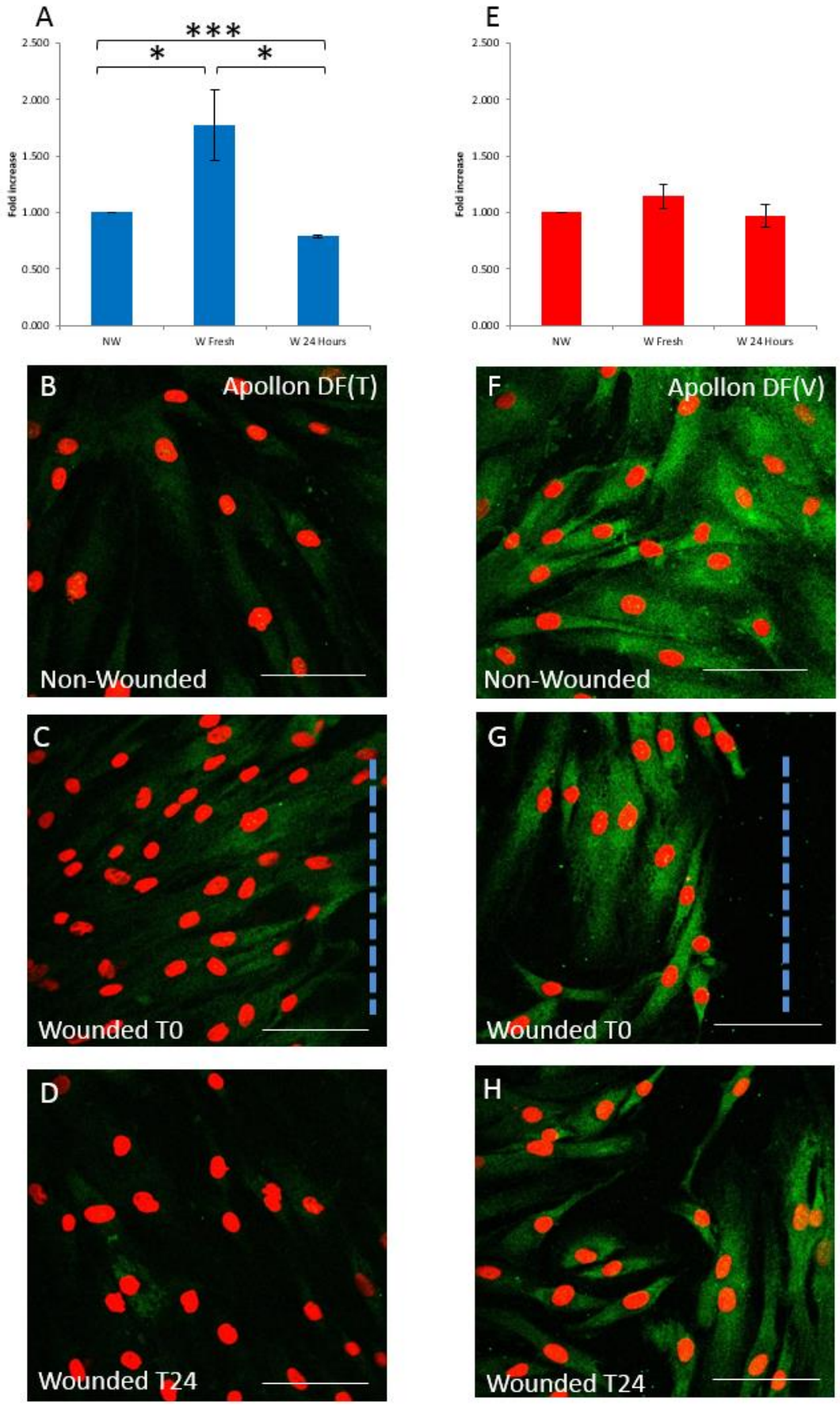
Immunofluorescent staining was carried out in matched DF(T) and DF(V) cultures to compare the expression of the IAPs, Apollon, cIAP2, NAIP and XIAP protein under different wounding conditions; quantification was via ImageJ analysis (Figure 3.10, Figure 3.12, Figure 3.13, and Figure 3.14 respectively).

3.3.7.1 Increased level of Apollon expression after immediate wounding

Expression of Apollon was located in the cytoplasm of the dermal fibroblasts (Figure 3.10). The expression of Apollon in unwounded and 24 hours following wounding was higher in DF(V) cells compared to matching DF(T) cells ($p<0.05$ and $p<0.005$ respectively) (Figure 3.11A), mirroring mRNA expression (Figure 3.7A), which showed that unwounded DF(V) cells expressed approximately twice the level of Apollon mRNA than corresponding DF(T).

By analysing the density of Apollon expression in DF(T) cells using ImageJ (Figure 3.10A), expression was significantly increased, ($p<0.05$) after immediate mechanical wounding, and then decreased to a level below the expression seen prior to wounding ($p<0.0005$). Representative photomicrographs for the immunolocalisation of Apollon in unwounded DF(T) cultures, immediately, and then 24 hours following mechanical wounding are shown in Figure 3.10B, Figure 3.10C and Figure 3.10D respectively.

Using ImageJ to analyse the density of Apollon in DF(V) cells (Figure 3.10E), no change in expression was detected immediately, nor 24 hours following mechanical wounding, in contrast to mRNA expression (Figure 3.8B). Figure 3.10F, Figure 3.10G and Figure 3.10H represents photomicrographs for Apollon in unwounded DF(V) cultures, immediately, and then 24 hours following mechanical wounding respectively.


A
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2.500
2.000
1.500
1.000
0.500
0.000
Fold increase
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W Fresh
W 24 Hours
E
2.500
2.000
1.500
1.000
0.500
0.000
Fold increase
NW
W Fresh
W 24 Hours
B
Apollon DF(T)
Non-Wounded
F
Apollon DF(V)
Non-Wounded
C
Wounded T0
G
Wounded T0
D
Wounded T24
H
Wounded T24

Figure 3.10: High expression of Apollon in DF(V) cells compared to DF(T) cells; increased expression of Apollon after wounding in DF(T) cells

*Immunofluorescent expression of Apollon in DF(T) and DF(V) monolayers under non-wounding and wounding conditions. A) Quantitated results from immunofluorescent images using ImageJ for images B-D. (B, C and D) Represent unwounded DF(T) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. E) Quantitated results from immunofluorescent images using ImageJ for images F-H. (F, G and H) Represent unwounded DF(V) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. Green staining – Apollon, red staining – DAPI, blue – DF(T), red – DF(V), dashed blue line – wounded scratch. Magnification bar 50 µm. n=3 donors, in duplicate wells at two points, * p<0.05, *** p<0.0005 (ANOVA-Bonferroni test).*

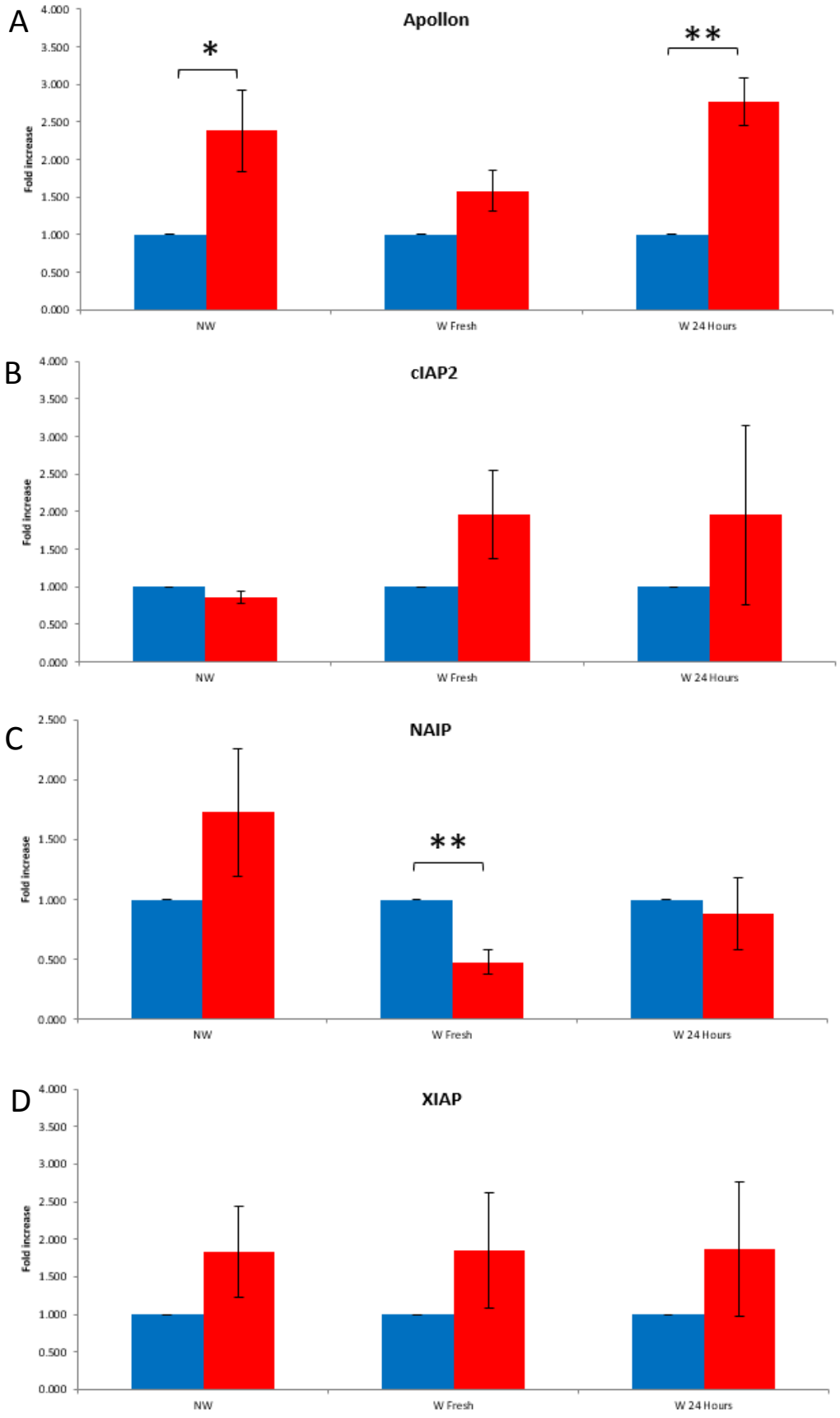

A
Apollon
*
**
Fold increase
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
NW
W Fresh
W 24 Hours
B
cIAP2
Fold increase
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
NW
W Fresh
W 24 Hours
C
NAIP
**
Fold increase
2.500
2.000
1.500
1.000
0.500
0.000
NW
W Fresh
W 24 Hours
D
XIAP
Fold increase
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
NW
W Fresh
W 24 Hours

Figure 3.11: Expression levels for the IAPs normalised against DF(T) cells

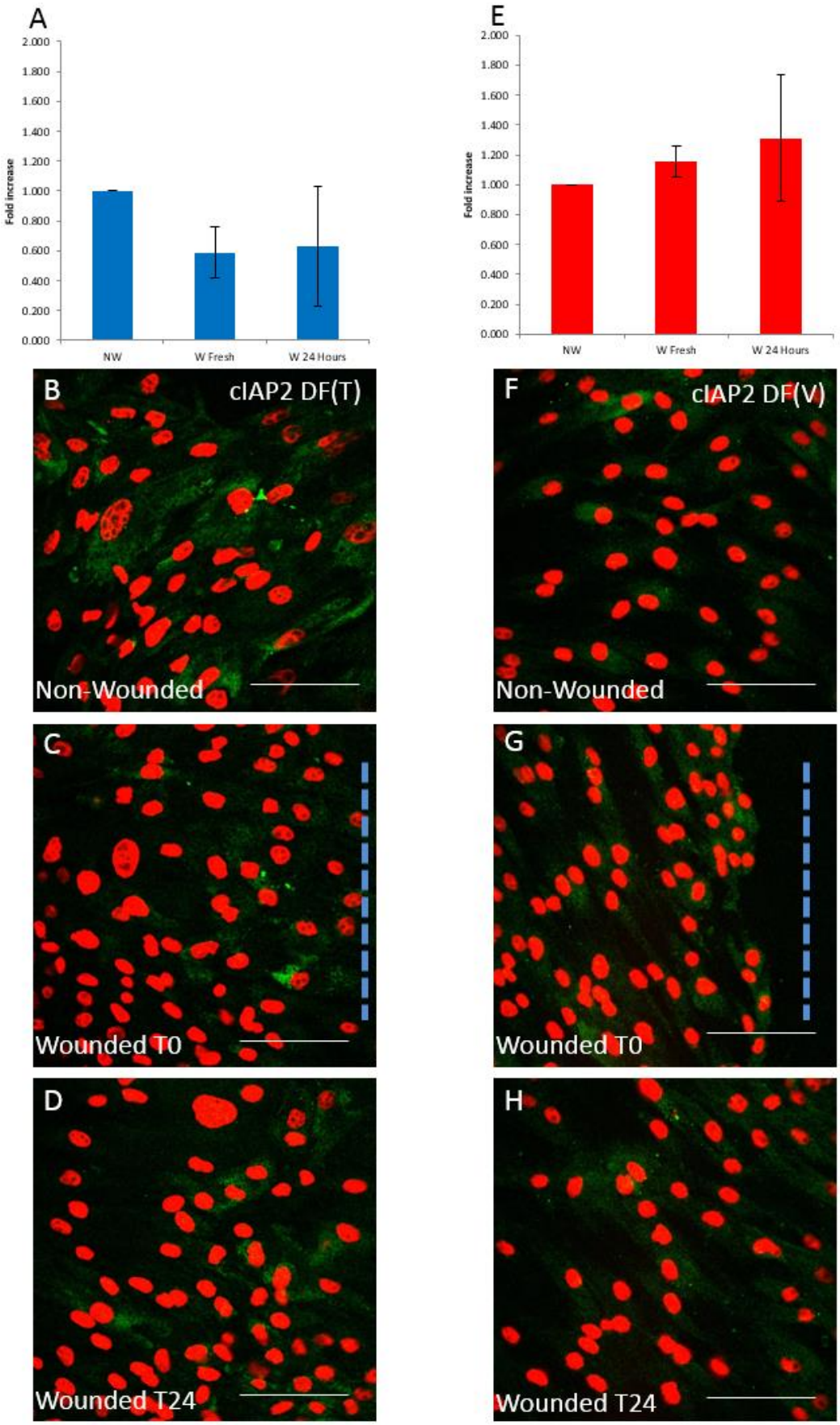
*Expression levels for DF(V) cells for each IAP and wounding condition was normalised against matching DF(T) cells. A) Expression level of Apollon protein in unwounded, immediate wounding and 24 hours after wounding. B) Expression level of cIAP2 protein in unwounded, immediate wounding and 24 hours after wounding. C) Expression level of NAIP protein in unwounded, immediate wounding and 24 hours after wounding. D) Expression level of XIAP protein in unwounded, immediate wounding and 24 hours after wounding. Red – DF(V), blue – DF(T). n=3 donors, (Student-T-test), * p<0.05, ** p<0.005.*

3.3.7.2 No changes in cIAP2 expression levels when dermal fibroblast monolayers were wounded in culture

Expression of cIAP2 was located in the cytoplasm of the dermal fibroblasts (Figure 3.12). cIAP2 protein expression levels under unwounded and mechanically wounded conditions were similar between DF(T) and matching DF(V) cultures (Figure 3.11B); while mRNA expression was higher in DF(V) cells compared to matching DF(T) cells (Figure 3.7A).

The expression of cIAP2 in DF(T) cultures did not change when cells were mechanically wounded, either at time point 0, or after 24 hours (Figure 3.12A). Figure 3.12B, Figure 3.12C and Figure 3.12D represents photomicrographs for cIAP2 in unwounded DF(T) cultures, immediately, and 24 hours following mechanical wounding respectively. This was in contrast to mRNA expression, when expression was decreased 24 hours following mechanical wounding (Figure 3.8A).

Figure 3.12E represents protein expression of cIAP2 in unwounded, immediately, and 24 hours following mechanical wounding in DF(V) cultures. In contrast to mRNA expression, which decreased after 24 hours of mechanical wounding (Figure 3.8B), no significant changes were seen in DF(V) cultures immediately or 24 hours following mechanical wounding. Representative photomicrographs for cIAP2 in unwounded DF(V) culture, immediately, and 24 hours following mechanical wounding are shown in Figure 3.12F, Figure 3.12G and Figure 3.12H respectively.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Fold increase
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1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
NW
W Fresh
W 24 Hours
E
Fold increase
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
NW
W Fresh
W 24 Hours
B
cIAP2 DF(T)
Non-Wounded
F
cIAP2 DF(V)
Non-Wounded
C
Wounded T0
G
Wounded T0
D
Wounded T24
H
Wounded T24

Figure 3.12: No change in cIAP2 expression when wounded

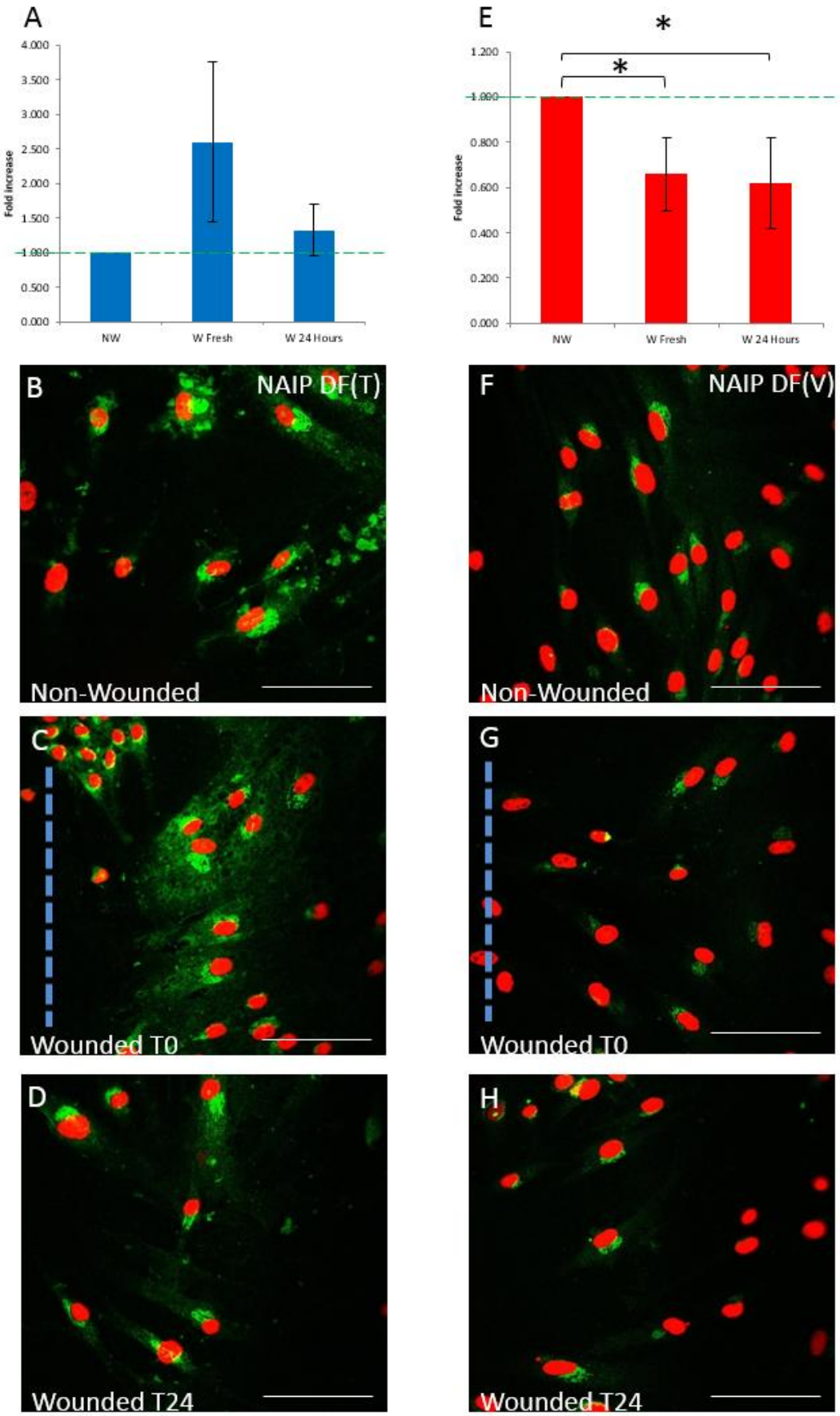
Immunofluorescent expression of cIAP2 in DF(T) and DF(V) monolayers under non-wounding and wounding conditions. A) Quantitated results from immunofluorescent images using ImageJ for images B-D. B, C and D) Represent unwounded DF(T) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. E) Quantitated results from immunofluorescent images using ImageJ for images F-H. F, G and H) Represent unwounded DF(V) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. Green staining – cIAP2, red staining – DAPI, blue – DF(T), red – DF(V), dashed blue line – wounded scratch. Magnification bar 50 µm. n=3 donors, in duplicate wells at two points.

3.3.7.3 Expression of NAIP in DF(V) cells decreased after mechanical wounding of cultured monolayer

The localisation of NAIP protein was confirmed just outside the nucleus of unwounded DF(T) and matching DF(V) cultures (Figure 3.13) using immunofluorescent staining, quantified using ImageJ. NAIP protein expression between DF(T) and DF(V) cells in unwounded and 24 hours following mechanical wounding were similar, with lower expression in DF(V) monolayers immediately after mechanical wounding, $p<0.005$, (Figure 3.11C).

In DF(T) cultures, NAIP protein expression did not significantly change immediately, or 24 hours following mechanical wounding (Figure 3.13A); this concurs with mRNA expression (Figure 3.8A). Representative photomicrographs for NAIP in unwounded DF(T) culture, immediately and 24 hours following mechanical wounding are shown in Figure 3.13B, Figure 3.13C and Figure 3.13D respectively.

In matching DF(V) cells, protein expression levels decreased immediately after mechanical wounding ($p<0.05$) and still remained low after 24 hours ($p<0.05$) (Figure 3.13E). Representative photomicrographs for NAIP in unwounded DF(T) cultures, immediately, and 24 hours following mechanical wounding are shown in Figure 3.13F, Figure 3.13G and Figure 3.13H respectively.


A
Fold increase
4.000
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W Fresh
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E
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Fold increase
1.200
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0.400
0.200
0.000
NW
W Fresh
W 24 Hours
B
NAIP DF(T)
Non-Wounded
F
NAIP DF(V)
Non-Wounded
C
Wounded T0
G
Wounded T0
D
Wounded T24
H
Wounded T24

Figure 3.13: Decrease in NAIP expression in DF(V) cells after wounding

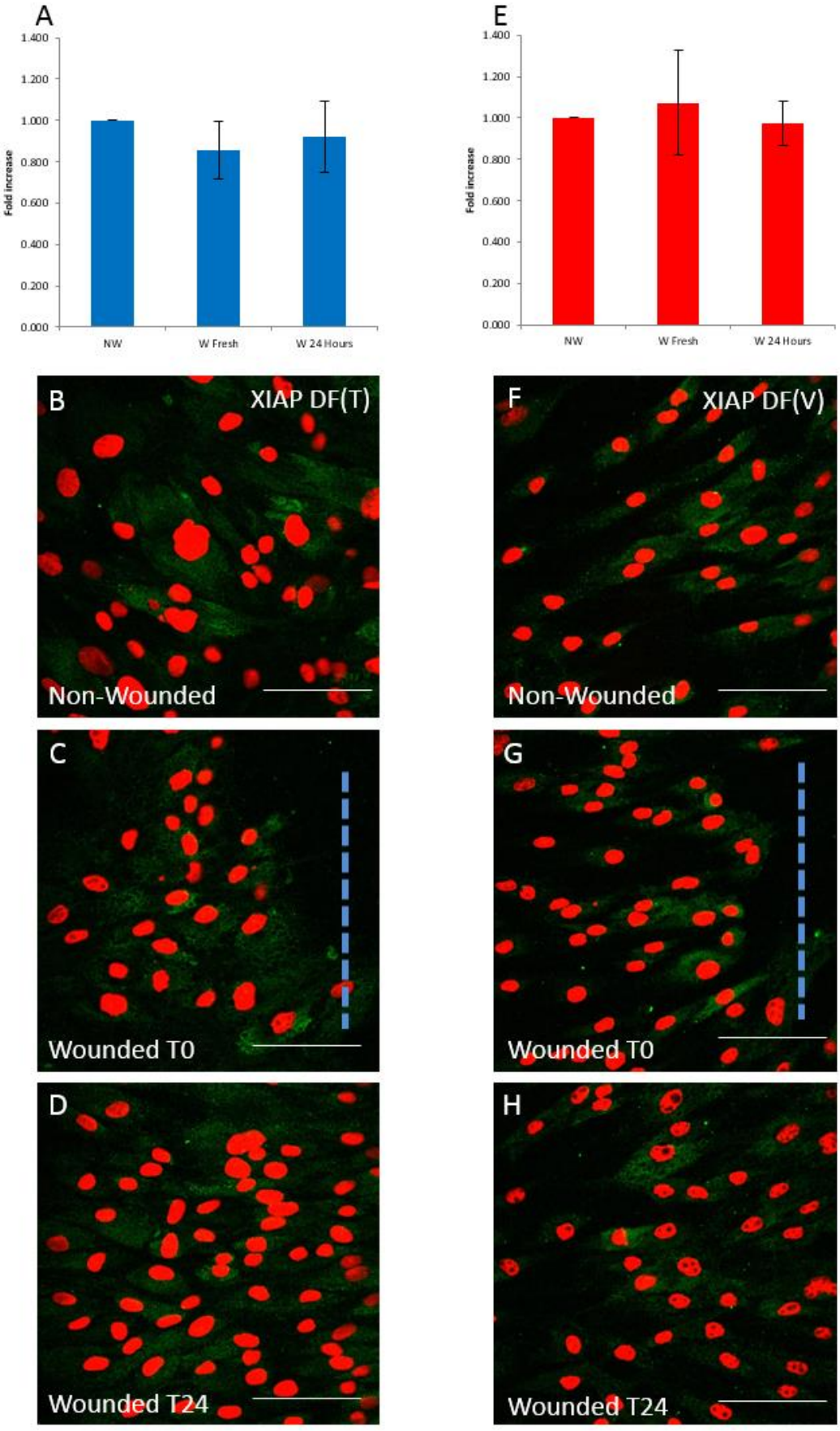
*Immunofluorescent expression of NAIP in DF(T) and DF(V) cell monolayers under non-wounding and wounding conditions. A) Quantitated results from immunofluorescent images using ImageJ for images B-D. (B, C and D) Represent unwounded DF(T) cultures, time zero (T0) immediately after wounding, and after 24 hours (T24) respectively. E) Quantitated results from immunofluorescent images using ImageJ for images F-H. (F, G and H) Represent unwounded DF(V) cultures, time zero (T0) immediately after wounding, and after 24 hours (T24) respectively. Green staining – NAIP, red staining – DAPI, blue – DF(T), red – DF(V), dashed blue line – wounded scratch. Magnification bar 50 µm. n=3 donors, * p<0.05 (ANOVA- Bonferroni test).*

3.3.7.4 No changes in XIAP protein expression levels in matching DF(T) and DF(V) cells when mechanically wounded

Localisation of XIAP protein was confirmed in the cytoplasm of DF(T) and matching DF(V) cultures (Figure 3.14) using immunofluorescent staining, quantified using ImageJ. Expression of XIAP protein between DF(T) and DF(V) cultures was the same in unwounded, immediately and 24 hours following mechanical wounding (Figure 3.11D). This reflected mRNA expression between matching DF(T) and DF(V) (Figure 3.7A).

In contrast to mRNA expression of XIAP in DF(T) cultures where expression decreased 24 hours following mechanical wounding (Figure 3.8A), protein expression levels did not change immediately, nor 24 hours following mechanical wounding (Figure 3.14A). Representative photomicrographs for XIAP in unwounded DF(T) cultures, immediately and 24 hours following mechanical wounding are shown in Figure 3.14B, Figure 3.14C and Figure 3.14D respectively.

Similarly with DF(T) cultures, mRNA expression did not concur with protein expression (Figure 3.8B); where mRNA expression increased 24 hours following mechanical wounding, no change in protein expression was observed (Figure 3.14E). Representative photomicrographs for XIAP in unwounded DF(T) culture, immediately and 24 hours following mechanical wounding are shown in Figure 3.14F, Figure 3.14G and Figure 3.14H respectively.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Fold increase
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B
XIAP DF(T)
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XIAP DF(V)
Non-Wounded
C
Wounded T0
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Wounded T0
D
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Wounded T24

Figure 3.14: No change in XAP expression in DF(T) and DF(V) cells after wounding

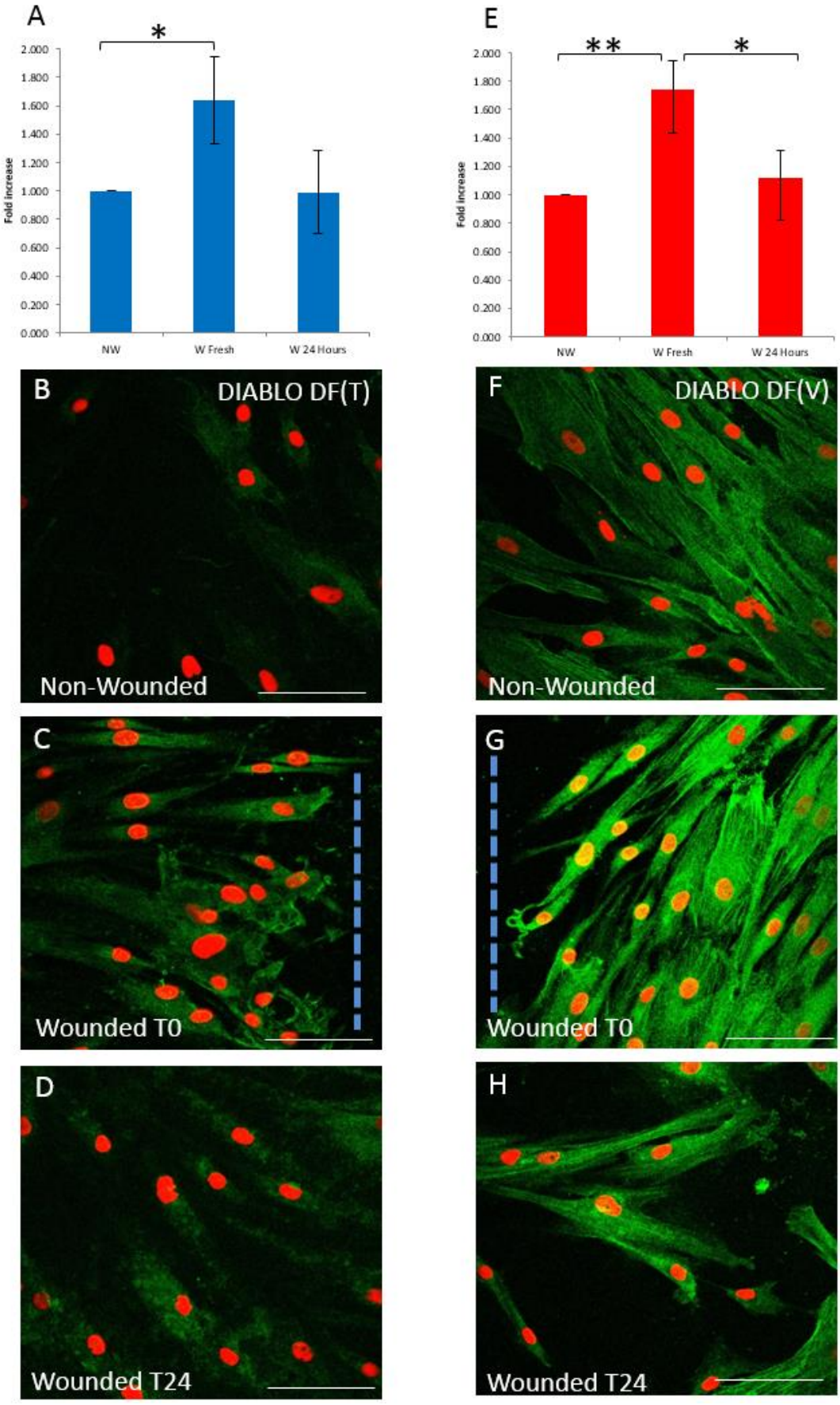
Immunofluorescent expression of XIAP in DF(T) and DF(V) cell monolayers under non-wounding and wounding conditions. A) Quantitated results from immunofluorescent images using ImageJ for images B-D. (B, C and D) Represent unwounded DF(T) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. E) Quantitated results from immunofluorescent images using ImageJ for images F-H. (F, G and H) Represent unwounded DF(V) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. Green staining – XIAP, red staining – DAPI, blue – DF(T), red – DF(V), dashed blue line – wounded scratch. Magnification bar 50 µm. n=3 donors.

3.3.8 Expression of DIABLO increased immediately after mechanical wounding, and returned to basal levels after 24 hours

The localisation of DIABLO protein was confirmed in the cytoplasm of DF(T) and matching DF(V) cultures with slight nuclear staining in DF(V) cells adjacent to the wound edge when immediately wounded, analysed via immunofluorescent staining and quantified using ImageJ (Figure 3.15).

Expression of DIABLO protein between DF(T) and matching DF(V) cells showed no difference in unwounded cells, with higher expression in DF(V) cells immediately and 24 hours following mechanical wounding ($p<0.05$) (Figure 3.16A). This data correlates with mRNA expression between DF(T) and DF(V) cells (Figure 3.7B).

In contrast to mRNA expression, which showed a decrease in DIABLO mRNA 24 hours following mechanical wounding in DF(T) and DF(V) cells (Figure 3.9A and Figure 3.9C respectively), protein expression increased immediately after mechanical wounding in DF(T) and DF(V) cultures ($p<0.05$ and $p<0.005$ respectively), returning to basal levels 24 hours following mechanical wounding (Figure 3.15A and Figure 3.15E respectively). Representative photomicrographs for the expression of DIABLO in DF(T) cultures (Figure 3.15B, Figure 3.15C and Figure 3.15D), and DF(V) cultures (Figure 3.15F, Figure 3.15G and Figure 3.15H) either unwounded, immediately, or 24 hours following mechanical wounding.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B
DIABLO DF(T)
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DIABLO DF(V)
Non-Wounded
C
Wounded T0
G
Wounded T0
D
Wounded T24
H
Wounded T24

Figure 3.15: Increased levels of DIABLO in both DF(T) and DF(V) cells after immediate wounding, with expression levels returning to non-wounded levels 24hours after wounding

*Immunofluorescent expression of DIABLO in DF(T) and DF(V) cell monolayers under non-wounding and wounding conditions. A) Quantitated results from immunofluorescent images using ImageJ for images B-D. (B, C and D) Represent unwounded DF(T) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. E) Quantitated results from immunofluorescent images using ImageJ for images F-H. (F, G and H) Represent unwounded DF(V) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. Green staining – DIABLO, red staining – DAPI, blue – DF(T), red – DF(V), dashed blue line – wounded scratch. Magnification bar 50 µm. n=3 donors, * p<0.05, ** p<0.005 (ANOVA-Bonferroni test).*

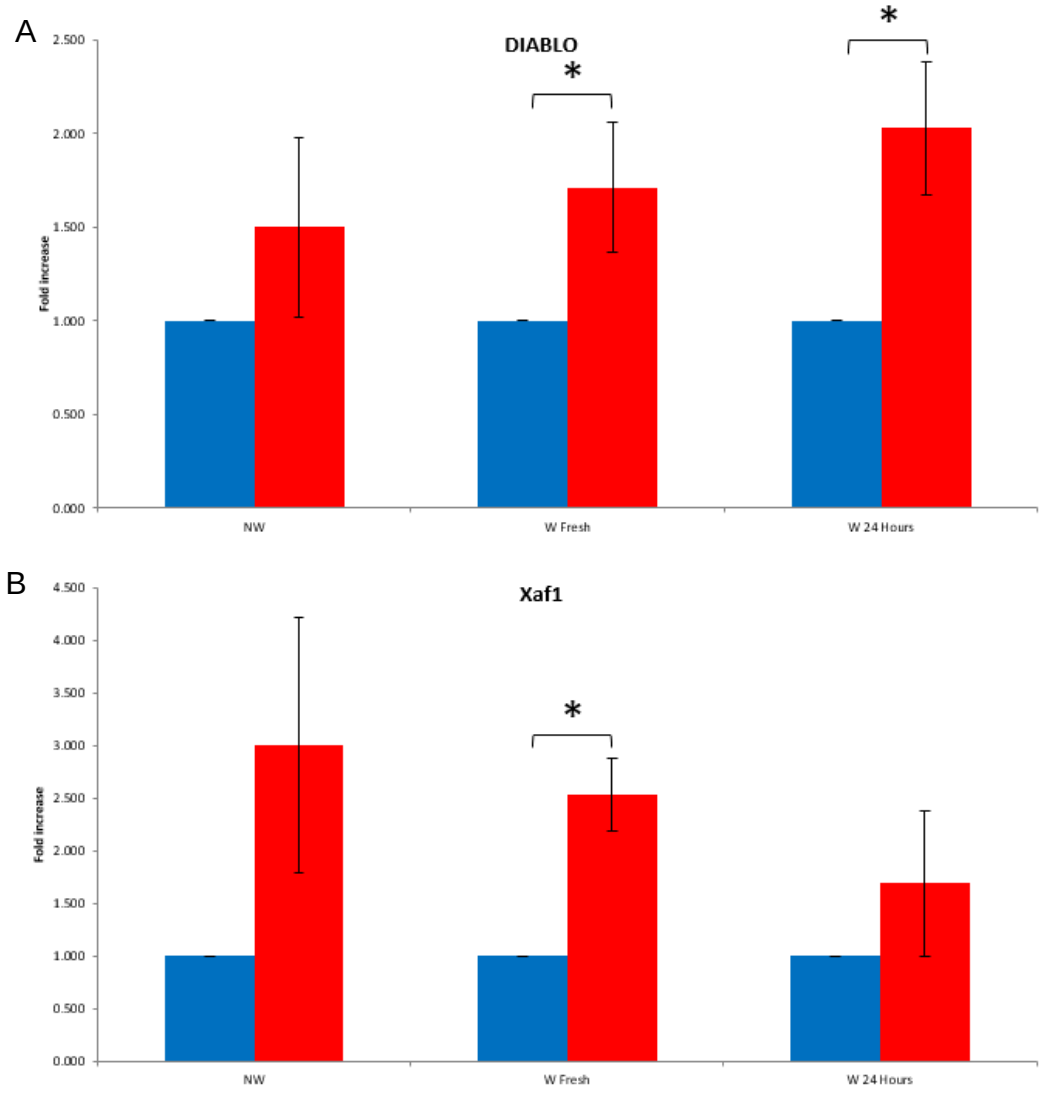


Figure 3.16: Expression levels for the IAP antagonist normalised against DF(T) cells

*Expression levels for DF(V) cells for each IAP antagonist and wounding condition normalised against matching DF(T) cells. A) Expression level of DIABLO protein in unwounded, immediate wounding and 24 hours after wounding. B) Expression level of Xaf1 protein in unwounded, immediate wounding and 24 hours after wounding. Red – DF(V), blue – DF(T). n=3 donors, Student-T test, * p<0.05.*

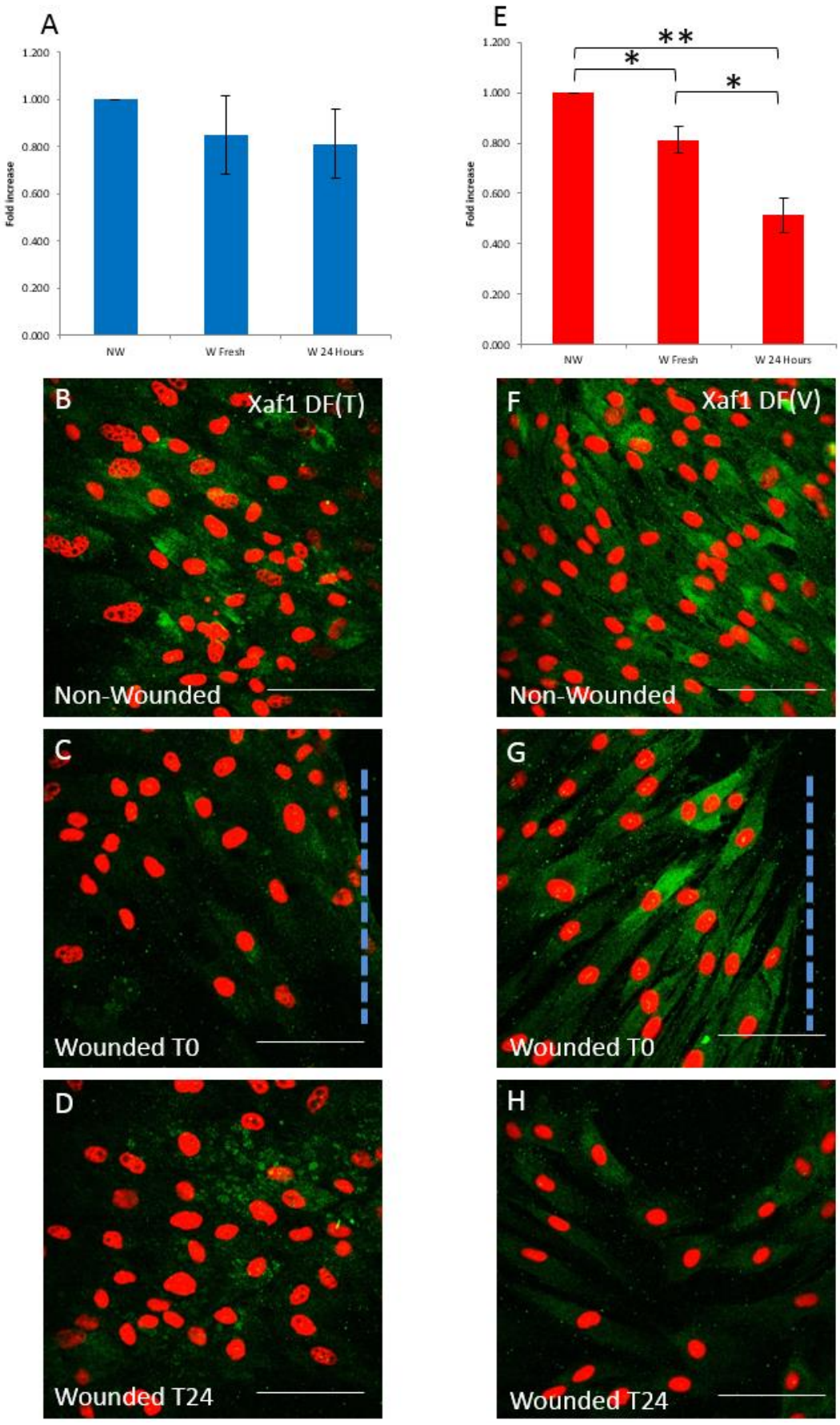
3.3.9 Continual decrease in Xaf1 expression after mechanically wounding only in DF(V) cultures

The localisation of Xaf1 protein was confirmed in the cytoplasm of DF(T) and matching DF(V) cultures via immunofluorescent staining and quantified using ImageJ (Figure 3.17).

When comparing the expression of Xaf1 protein between DF(T) and DF(V) cells under unwounded and mechanically wounded conditions, Xaf1 was higher in DF(V) cells immediately after mechanical wounding ($p<0.05$) (Figure 3.16B). However, no differences were seen in unwounded cells and 24 hours following mechanical wounding, which concurs with mRNA expression (Figure 3.7C)

Xaf1 protein expression in DF(T) cells did not change following mechanical wounding (Figure 3.17A), this correlates with mRNA expression, which showed no change in Xaf1 24 hours following mechanical wounding (Figure 3.9B). Representative photomicrographs for Xaf1 protein expression in unwounded, immediately, or 24 hours following mechanical wounding are given in Figure 3.17B, Figure 3.17C and Figure 3.17D respectively.

In contrast, protein expression for Xaf1 in matching DF(V) cells decreased immediately following mechanical wounding ($p<0.05$), and continued to further decrease 24 hours following mechanical wounding ($p<0.005$) (Figure 3.17E). The decrease in protein levels 24 hours following mechanical wounding concurs with mRNA expression (Figure 3.9D). Representative photomicrographs for Xaf1 protein expression in unwounded, immediately and 24 hours following mechanical wounding are given in Figure 3.17F, Figure 3.17G and Figure 3.17H respectively.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W Fresh
W 24 Hours
B
Xaf1 DF(T)
Non-Wounded
F
Xaf1 DF(V)
Non-Wounded
C
Wounded T0
G
Wounded T0
D
Wounded T24
H
Wounded T24

Figure 3.17: Continual decrease in Xaf1 expression in DF(V) immediately and 24 hours after wounding

*Immunofluorescent expression of Xaf1 in DF(T) and DF(V) cell monolayers under non-wounding and wounding conditions. A) Quantitated results from immunofluorescent images using ImageJ for images B-D. B, C and D) Represent unwounded DF(T) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. E) Quantitated results from immunofluorescent images using ImageJ for images F-H. F, G and H) Represent unwounded DF(V) cultures, time zero (T0) after immediately wounding, and after 24 hours (T24) respectively. Green staining – Xaf1, red staining – DAPI, blue – DF(T), red – DF(V), dashed blue line – wounded scratch. Magnification bar 50 µm. n=3 donors, * p<0.05, ** p<0.005 (ANOVA-Bonferroni test).*

3.4 Discussion

Although previously comparisons have been made between reticular and papillary dermal fibroblasts (Sorrell et al., 2004; Sorrell and Caplan, 2004), and dermal fibroblasts from different anatomical sites (Stevenson, 2007), this is the first study to compare dermal fibroblasts derived from matching terminal and vellus hair bearing skin from a similar anatomical region of the same individual.

3.4.1 Morphology of dermal fibroblasts

A number of differences were observed between matching cultures of dermal fibroblasts derived from terminal hair bearing skin (DF(T)) and vellus hair bearing skin (DF(V)) of the same donor. In the present study, a comparison of morphology revealed differences in terms of size and complexity/granularity (the number of cytoplasmic granules and membrane size), where DF(V) cells were smaller and more granular than matching DF(T) cells (Figure 3.4). A study by Chipev and Simon (2002) demonstrated a difference in cell morphology between fibroblasts cultured from different anatomical regions such as keloid skin (a scar with overactive fibroblasts causing excessive deposition of ECM components), palmar (skin obtained from the palm region), plantar (skin from the soles of the feet) and nonplantar skin (the rest of the skin region which contains vellus hair follicles). This group reported that the size of palmer fibroblasts were smaller, and that keloid and nonplantar fibroblasts were more granular than palmar and plantar fibroblasts. They suggested that the difference in morphology was due to interactions with different signalling pathways, in particular to proteins involved in the TGFβ1 signalling pathway. When they compared granularity and TGFβ1 receptor II expression between the different dermal fibroblasts, there was a correlation between higher granularity of cells and higher receptor expression. Although they did not look at the difference between reticular and papillary dermal fibroblasts, a later study by Sorrell et al. (2004) showed that structurally in a cell monolayer culture, there is a difference in the distribution and layout of the fibroblasts; further indicating that fibroblasts from different regions may possess different properties that could affect cell characteristics.

3.4.2 Migration of dermal fibroblasts

A study by Stevenson (2007) compared migration of human female dermal fibroblasts from three different anatomical regions, scalp, abdomen and breast. In a scratch wound assay it was shown that after 48 hours, abdominal and scalp dermal fibroblasts had migrated faster than breast dermal fibroblasts. However, at the earlier time point of 24 hours, there was no significant difference. Using the same scratch wound assay, scalp dermal fibroblast migration was compared to dermal papilla and dermal sheath cell migration; no difference was detected in migration 24 hours and 48 hours after scratching. In the present study (Figure 3.5), there was no significant difference in the migration of DF(T) compared to DF(V) cultures. This demonstrates that whilst potentially fibroblasts from different anatomical regions migrate at different rates, fibroblasts from vellus and terminal hair bearing skin from a similar anatomical region (face/scalp) from the same donor migrate at a similar rate under basal conditions.

Cell migration *in vivo* during wound healing will occur in different 3D directions. Since the experimental design of this study used a monolayer, the assessment of cell migration was in 2D. There are studies that have compared the migration of fibroblasts in 2D and 3D models (Hakkinen et al., 2011; Rhee, 2009). Hakkinen et al. (2011) showed that there was a difference in direction and velocity of cell migration in 3D models compared to equivalent 2D models. This is important as it was hypothesised by McDougall et al. (2006) that collagen deposition is parallel to fibroblast migration, and therefore differences in DF(T) and DF(V) migration may affect how a scar is formed.

3.4.3 Dermal fibroblast proliferation

From the study by Chipev and Simon (2002), which compared the morphology of fibroblasts from different anatomical regions, it was observed that fibroblasts that had higher granularity expressed higher levels of the TGFβ1 receptor, which is important in driving differentiation of dermal fibroblasts into myofibroblasts, and is also important in dermal fibroblast proliferation (Jutley

et al., 1993; O'Leary et al., 2004). In the study by Chipev and Simon (2002), cells that were more granular showed a lower proliferation rate, as assessed by measuring incorporations of ^{3}H-thymidine to measure DNA synthesis.

The data generated using FACS (Figure 3.4) comparing DF(T) and matching DF(V) cells revealed a higher granularity of DF(V) cells compared to corresponding DF(T) cells. This could be important in maintaining the vellus hair follicle skin phenotype. TGFβ1 shortens anagen and induces catagen in the hair cycle (Ohnemus et al., 2006). Therefore, if the more granular vellus hair bearing dermal fibroblasts produce more TGFβ1 compared to equivalent terminal hair bearing dermal fibroblasts, the anagen phase may be shortened, and hair follicles prevented from differentiating into terminal hair follicles, keeping them in the vellus state. This theory is supported by results obtained from Stevenson (2007), which showed that dermal fibroblasts derived from breast skin secreted TGFβ1 into the culture medium, while dermal fibroblasts derived from the scalp, dermal sheath and the dermal papilla did not.

However, in contrast to the present study, where DF(V) cells showed a higher level of granularity, their rate of proliferation was also higher. Stevenson (2007), using ^{3}H-thymidine uptake to measure DNA synthesis, showed that dermal fibroblasts cultured in serum free medium derived from breast skin proliferated at a higher rate compared to those from abdominal and scalp skin, while there was no difference in the rate of proliferation between scalp and abdominal dermal fibroblasts. The breast fibroblasts were derived from younger donors (mean age - 29 years), whilst both abdominal and scalp fibroblasts were from older donors (mean age - 52 and 49 years respectively), which may contribute to the difference in proliferation. In the present study, the mean age of the donors used was 56 years, and each comparison between DF(T) and DF(V) cells were cultured from matching donors. Also, due to the duration in which proliferation was measured (12 days), serum needed to be added to maintain viability, however in the study by Stevenson (2007), no serum was added, which would also influence the proliferation rate. In the same study by Stevenson (2007), dermal fibroblasts derived from breast skin

secreted TGFβ1, while in dermal fibroblasts from scalp skin, there was no secretion which could be an influential factor in cell proliferation.

TGFβ1 may not only potentially influence cell proliferation of DF(V) cells, but may also influence fibroblast differentiation to myofibroblasts causing more scarring with a higher number of fibroblasts in the region expressing α-SMA for contraction. This reflects previous studies stating that skin from hairy regions heal better and with less scarring compared to those from non hairy regions (Jahoda and Reynolds, 2001; Martinot et al., 1994). An interesting study to verify this claim, would be to compare the secretion of TGFβ1 using an ELISA assay and expression of α-SMA either via immunohistochemistry or mRNA expression in DF(T) and matching DF(V) cells and look at the potential contraction ability using a collagen gel.

3.4.4 Expression of IAPs in wound healing

Recently, there has been some interest in the role of IAPs in wound healing. Fuchs et al. (2013) in an *in vivo* study, demonstrated that XIAP was expressed in the skin and hair follicles of mice. Its importance in wound healing was demonstrated by gene knockout, which confirmed that in the absence of XIAP function, wound healing was compromised.

In the present study, mRNA expression of XIAP was similar between unwounded DF(T) and matching DF(V) cells (Figure 3.7). However, 24 hours following mechanical wounding, expression of XIAP mRNA had decreased in DF(T) cells, whilst it had increased in DF(V) cells (Figure 3.8), although further analysis of the protein expression between DF(T) and DF(V) cells following mechanical wounding did not show a difference in expression (Figure 3.11). The study by Fuchs et al. (2013) demonstrated in mice that the hair follicle bulge (niche for epidermal stem cells) of telogen hair follicles expressed XIAP, and that improved wound healing was attributed to the increase in the hair follicle stem cells being protected against apoptosis. The knockdown of an XIAP antagonist (Sept4/Arts) improved wound healing, while with the knockdown of XIAP, re-epithelialisation and regeneration of the mouse skin

was compromised. The increase of XIAP mRNA in DF(V) cells 24 hours following mechanical wounding, suggests that dermal fibroblasts are protected against apoptosis within the first 24 hours. This may be linked to the higher rate of cell proliferation in DF(V) compared to DF(T) cells. The cells were incubated in low serum concentrations over a 12 day period, and other studies have shown that low serum concentration significantly reduces proliferation (Kniss and Burry, 1988; Maltseva et al., 2001). Low serum rendered rat astrocytes quiescent (Kniss and Burry, 1988). While fibroblasts derived from rabbit cornea cultured in 10% and 1% FBS for 2 days, showed a decrease in cell proliferation in fibroblasts cultured in 1% FBS compared to 10% FBS as detected by a BrdU assay (Maltseva et al., 2001). In contrast, XIAP decreased within the first 24 hours in DF(T) cells (Figure 3.8), which could explain why they did not proliferate well under low serum concentration. Cell viability would confirm the effect of low serum on DF(T) and DF(V) cell proliferation and apoptosis, for example using a TUNEL and BrdU assay to analyse apoptosis and proliferation respectively. The difference between the changes in the expression of mRNA and protein may be due to a time difference, the duration from which XIAP mRNA is transcribed to protein is not known, therefore either more time is needed for protein expression to be modulated, or XIAP mRNA is processed immediately after wounding. This could also be due to the necessity of a quick response; mRNA levels are transcribed but not converted into proteins until needed. Therefore, changes in protein and mRNA expression need to be analysed at more frequent time intervals.

Xaf1 is a direct XIAP target, binding to XIAP preventing XIAP from functioning (Liston et al., 2001). Expression of Xaf1 mRNA was the same in DF(T) and DF(V) cells. In mechanically wounded DF(T) cells, mRNA expression remained constant, whilst in DF(V) cells, expression decreased (Figure 3.9). Similarly, protein Xaf1 expression remained constant in DF(T) under wounded conditions, whilst expression decreased in DF(V) cells immediately and 24 hours following mechanical wounding (Figure 3.17). Therefore, even though protein expression levels of XIAP remained constant after wounding in DF(V)

cells, the activity of XIAP is probably enhanced following the reduction in the expression of Xaf1.

In the mouse endothelial cell line (MS1), upregulation of Xaf1 caused a decrease in cell proliferation (Qiao et al., 2008). This may explain the higher proliferation rate of DF(V) cells, with reduced expression of Xaf1. While the change in the expression of Xaf1 may be due to the stress of mechanical wounding, low serum concentrations over a 12 day period may also subject the cells to stress. To determine whether the expression of Xaf1 is altered with stress inducers, DF(T) and DF(V) cells should be compared under different conditions such as incubation with TNFα or hydrogen peroxide and exposure to UV. To determine whether changes in the expression of Xaf1 modulates cell proliferation via its interaction with XIAP, embelin, a XIAP inhibitor can be added to DF(T) and DF(V) cell cultures, and mRNA expression and protein expression analysed under different wounding conditions. Another method which may be used, would be to silence the XIAP gene using siRNA and analyse cell proliferation under different conditions.

NAIP has been shown to have a protective function in neurones in rats against ischemia (Xu et al., 1997a). Messenger RNA expression of NAIP was higher in DF(V) cells compared to matching DF(T) cells, however when mechanically wounded, expression of NAIP did not change in either DF(T) nor DF(V) cells (Figure 3.8). Protein expression for NAIP in DF(T) cells did not change when mechanically wounded, however, protein expression in DF(V) cells was reduced immediately following mechanical wounding, and still remained low after 24 hours (Figure 3.13). NAIP does not contain a ring domain, which is generally targeted for ubiquitination and degradation of proteins (Cheung et al., 2008). Depending on the amino acids found in the N-terminal, the protein can be short lived (30 minutes) or long lived (30 hours) (Lodish et al., 2000). To inhibit apoptosis by binding to caspase 9, ATP is required (Davoodi et al., 2010b), which causes a conformational change to the protein (Davoodi et al., 2004). Expression of NAIP protein could decrease and remain low after 24 hours following mechanical wounding due to NAIP binding to caspase 9 to

prevent apoptosis causing a conformational change therefore the antibody might not be able to bind to the protein. Since the levels of NAIP are initially low, they might not be important in the initial wound healing response. However, this could also be due to the fact that NAIP mRNA is more stable and therefore high quantities are not required for a higher rate of protein synthesis.

The expression of Apollon mRNA in both DF(T) and DF(V) was much higher than XIAP, NAIP and cIAP2 (Figure 3.7), suggesting that in dermal fibroblasts Apollon may have an important role. A study in mice with a mutant Apollon gene reported that the mice died between day 11.5 and 14.5 of embryonic development (Hitz et al., 2005). This was due to the lack of proliferation of spongiotrophoblast in placental development, indicating that Apollon is important in cell division. Another study, but *in vitro*, demonstrated that in the endothelial cell line (LoVo), blocking of Apollon, inhibited cell proliferation (He et al., 2011). Messenger RNA and protein expression of Apollon was higher in DF(V) cells than corresponding DF(T) cells (Figure 3.7 and Figure 3.10 respectively); if Apollon does indeed affect cell division and proliferation, it could explain the increased rate of proliferation in DF(V) cells compared to matching DF(T) cells. To establish the effect of Apollon on cell proliferation, a comparison of the proliferation of DF(T) and DF(V) cells should be carried out following gene silencing of Apollon.

Expression of Apollon mRNA did not change 24 hours following mechanical wounding in DF(T) cells, however, it decreased in DF(V) cells (Figure 3.8). Protein expression in DF(T) cells increased immediately following mechanical wounding, returning to unwounded level of expression after 24 hours, whilst no change in protein expression was seen in DF(V) cells (Figure 3.10). This could be due to a specific expression level needed to respond to an inflicted wound. Since the levels of Apollon protein were already higher in unwounded DF(V) cells compared to corresponding DF(T) cells, the increase in Apollon protein expression immediately following mechanical wounding in DF(T) cells brought protein levels to those seen in DF(V) cells. Which would suggest that

in mechanically wounded cells Apollon is needed at a specific level to protect DF(T) and DF(V) cells.

cIAP2 is important in regulating apoptosis in epithelial cells (Roscioli et al., 2013). cIAP2 protects cells against damage and apoptosis when stressed, for example, in rat liver, cIAP2 expression increased when hepatocytes were exposed to bile duct ligation (Schoemaker et al., 2003). Expression of cIAP2 mRNA was higher in DF(V) cells compared to matching DF(T) cells (Figure 3.7). When mechanically wounded, mRNA expression decreased in both DF(T) and DF(V) cells (Figure 3.8), while protein expression remained the same, and was similarly expressed in DF(T) and DF(V) cells (Figure 3.12). The reduction of mRNA expression 24 hours after mechanical wounding would suggest that this protein was not important in the proliferation and migration of dermal fibroblasts following mechanical wounding, at least not immediately.

There is increasing evidence to suggest that another IAP, Survivin, may potentially have another role apart from inhibiting apoptosis (Botchkareva et al., 2007; Dallaglio et al., 2012; Dallaglio et al., 2009). Survivin has been associated with cell proliferation in keratinocytes; while over expression of Survivin does not modulate keratinocyte proliferation, down regulation of Survivin impairs the ability of keratinocytes to proliferate (Thomas et al., 2007). Therefore, potentially, changes in the expression of IAP mRNA and proteins in dermal fibroblasts could modulate cell proliferation. Since DF(V) cells proliferated faster than corresponding DF(T) cells, and only Apollon protein expression was higher in DF(V) cells compared to DF(T) cells in unwounded conditions (Figure 3.11), this could indicate a potential role for Apollon in dermal fibroblast proliferation. Silencing the Apollon gene in DF(V) cells and analysing DF(V) cell proliferation would establish how much Apollon can influence cell proliferation.

DIABLO is an IAP antagonist (Verhagen et al., 2000), it is mostly associated with cIAP1, cIAP2 and XIAP, however it prevents all IAP interactions with caspase 9, thereby promoting apoptosis. Messenger RNA expression of

DIABLO was higher in DF(V) cells compared to DF(T) cells (Figure 3.7). When wounded, mRNA expression decreased in both DF(T) and DF(V) cells (Figure 3.9), suggesting it is downregulated. Its protein expression increased immediately following mechanical wounding, decreasing to unwounded levels after 24 hours in both DF(T) and corresponding DF(V) cells. The immediate increase in DIABLO protein could suggest that it is functioning to regulate other IAP expressions, to allow cell death to occur in damaged cells, but is not required after 24 hours. Using a TUNEL assay on wounded cells in correlation with DIABLO antibody, will highlight whether cells undergoing apoptosis also express DIABLO. The increase in expression could however indicate that DIABLO might not only function to antagonise IAPs, since *in situ*, DIABLO expression was high in the epidermis, dermis and hair follicle of both terminal and vellus hair bearing skin (Figure 2.9 and Table 2.8). Although protein expression for DIABLO in unwounded cells was similar between DF(T) and DF(V) cells, when wounded, DIABLO was higher in DF(V) cells. This could indicate that DIABLO plays a more crucial role in DF(V) cells in regulating IAP expression and preventing apoptosis.

	DF(V) cells compared to DF(T) cells
Size	Smaller and more granular
Proliferation	Higher
Migration	No difference

mRNA expression of:	DF(V) cells compared to DF(T) cells
NAIP	Higher
cIAP2	Higher
Apollon	Higher
XIAP	Same
DIABLO	Higher
Xaf1	Same

Effect of wounding cells on:	DF(V) cells compared to DF(T) cells
NAIP	Higher
cIAP2	Higher
Apollon	Higher
XIAP	Higher
DIABLO	Higher
Xaf1	Same

Table 3.4: Comparison between DF(T) and DF(V) cells

A comparison between DF(V) and DF(T) cells in size, proliferation and migration, and mRNA expression of the IAPs NAIP, cIAP2, Apollon and XIAP, their antagonists DIABLO and Xaf1 under wounded and non-wounded conditions.

3.5 Conclusion

This chapter demonstrates there are significant differences between dermal fibroblasts cultured from the same region of skin that contains either terminal (DF(T)) or vellus (DF(V)) hair follicles of the same donor. Morphologically, DF(V) cells are smaller and more complex than DF(T). While DF(T) and DF(V) cells migrate at a similar rate in culture, DF(V) cells proliferate at a faster rate than corresponding DF(T) cells.

The expression of the IAPs NAIP, cIAP2, XIAP and Apollon and the IAP antagonists DIABLO and Xaf1 were shown to be present in both DF(T) and DF(V) cells at both transcriptional and protein levels.

At the mRNA level, expression of NAIP, cIAP2, Apollon and DIBALO was higher in DF(V) cells compared to DF(T) cells, however, at the protein level, only the expression of Apollon was higher in DF(V) cells.

Mechanical wounding of the cell monolayer increased mRNA expression of XIAP in DF(V) cells, but decreased expression in corresponding DF(T) cells. Mechanical wounding decreased cIAP2 mRNA expression in both DF(T) and DF(V) cells. The expression of Apollon mRNA was only decreased in DF(V) and not DF(T) cells following mechanical wounding. DIABLO mRNA expression decreased in both DF(T) and DF(V) cells, whilst only DF(V) cells showed a reduction in Xaf1 mRNA expression following mechanical wounding.

In contrast, mechanical wounding increased the protein expression of Apollon in DF(T) and decreased the protein expression of NAIP in DF(V) cells; there was no change in the protein expression of any of the other IAPs in either DF(T) nor DF(V) cells. Protein levels of DIABLO increased immediately following mechanical wounding, but returned to unwounded levels 24 hours later in both DF(T) and DF(V) cells, while the expression of the specific XIAP inhibitor Xaf1 was reduced only in DF(V) cells.

In summary, this chapter has demonstrated there are significant differences in morphology, proliferative potential, IAP expression and changes in IAP

expression in a scratch wound assay, in a direct comparison of dermal fibroblasts cultured from the same region of skin that contain terminal hair follicles (scalp) and vellus hair follicles (facial) of the same donor.

Chapter 4

A Comparison of the Effect of 17β-Oestradiol on Cultured Human Dermal Fibroblasts Derived from Terminal and Vellus Hair Bearing Skin

4 A Comparison of the Effect of 17β-Oestradiol on Cultured Human Dermal Fibroblasts Derived from Terminal and Vellus Hair Bearing Skin

4.1 Introduction

As mentioned in the previous chapters, IAPs have been implicated in oestrogen responses, in particular XIAP. There are some studies that have linked the regulation of IAPs by 17β-oestradiol. A study by Leblanc et al. (2003) demonstrated that in rat endometrium cells 17β-oestradiol altered XIAP and DIABLO levels, XIAP increased while DIABLO expression was reduced. Another study by Siegel et al. (2011) showed that XIAP is gender specific, in that the levels of XIAP in mice brain tissue was higher in females compared to age matched males. However, in this study, ovariectomised mice, treated with 17β-oestradiol showed no difference in XIAP levels to ovariectomised mice treated with oil. No other studies have looked at the effect of 17β-oestradiol on other IAP levels in skin. Since there has been some link between treatment with 17β-oestradiol and IAP expressions, it would be important to investigate if there are any differences in oestrogen receptor expression and how they may be affected by 17β-oestradiol between DF(T) and DF(V) cells. In dermal fibroblasts, 17β-oestradiol has been implicated in increasing dermal fibroblast proliferation (Son et al., 2005; Stevenson, 2007; Stevenson et al., 2005; Stevenson and Thornton, 2007).

4.1.1 Expression of oestrogen receptors in skin and hair follicles

Thornton et al. (2003b) demonstrated by immunohistochemistry that ERβ was the predominant oestrogen receptor expressed in male and female androgen independent scalp skin. Both ERα and ERβ were expressed in the sebaceous glands and in the papillary dermis, with stronger expression of ERβ. Nuclear staining for ERβ was detected in the bulb region of the hair follicle and also in the outer root sheath and the bulge region. Later, Kwon et al. (2004) in a comparison of male human androgen dependant and independent scalp using PCR and immunofluorescent staining, showed that ERα and ERβ were

expressed in the dermal papilla cells in both hair follicle types, with higher expression of both ERα and ERβ in the androgen independent hair follicle. A study by Qiao et al. (2012) demonstrated that in human foreskin, both ERα and ERβ were expressed, with expression of ERα in the basal levels of the epidermis. Another study by Jang et al. (2010), also demonstrated the expression of oestrogen receptor in the cheek skin of women.

In postmenopausal women, oestrogen levels decrease, skin becomes thinner, with reduced collagen content in the dermis and decreased skin elasticity (reviewed by Thornton (2013)); while replacement of oestrogen reverses the effects. An immunohistochemical study comparing oestrogen receptor expression of young and old donors in both sexes, showed that there was a significant decrease in ERβ expression in the epidermis of adults aged over 70 years (Inoue et al., 2011).

Topical treatment of human skin with 17β-oestradiol increases collagen synthesis, and epidermal keratinocyte proliferation (Son et al., 2005). While type I and III collagen decreases in the dermis of female skin 5 years after menopause (by up to 30%) (Affinito et al., 1999); the ratio of type III:type I collagen is also reduced. Topical 17β-oestradiol treatment increases the expression of the TGFβ2 receptor, which has been associated with dermal fibroblast proliferation and collagen secretion (Son et al., 2005).

4.1.2 Effect of 17β-oestradiol on wound healing

Oestrogen has been well established to have a positive effect during wound healing by enhancing re-epithelialisation and collagen deposition in both human and mouse skin (Ashcroft et al., 1997a; Campbell et al., 2010).

In the proliferative phase of wound healing, there was a delay in wound re-epithelialisation with reduced oestrogen (Ashcroft et al., 1997a), this was observed by comparing wound healing from pre- and post-menopausal women, this effect was reversed with the replacement of oestrogen via hormone replacement therapy (HRT). In aged donors not taking HRT, TGFβ1

decreased compared to young donors. Donors taking HRT, however showed an increase in TGFβ1, suggesting that 17β-oestradiol stimulates the production of TGFβ1. Due to the increase in TGFβ1 an increase in collagen production was seen in women taking hormone replacement compared to those deficient in oestrogen (Ashcroft et al., 1997a). In both male and female geriatrics, topical oestrogen increased collagen production, however, there was higher collagen deposition in female compared to males (Ashcroft et al., 1999).

Cell migration is an important aspect of wound healing. Two important cell types required to migrate in wounds are the dermal fibroblasts and the epidermal keratinocytes. A study by Stevenson et al. (2008b) showed that dermal fibroblasts derived from female facelift donors had an increased rate of cell migration when incubated with 17β-oestradiol; however, this effect was not seen with corresponding dermal sheath and dermal papilla cells. Further studies demonstrated that the increase in dermal fibroblast migration was seen with an ERα agonist, but not with an ERβ agonist. Interestingly dermal fibroblast migration in the presence of the ERα agonist was significantly higher than with 17β-oestradiol alone, indicating the importance of the ERα in dermal fibroblast migration.

TGFβ1 secretion increased in dermal fibroblasts that were incubated with 17β-oestradiol (Stevenson et al., 2008a). When cells were mechanically wounded, TGFβ1 secretion also increased, however, when mechanically wounded cells were treated with 17β-oestradiol, it inhibited TGFβ1 secretion. Indicating that 17β-oestradiol controls collagen synthesis and dermal fibroblast proliferation in wounded conditions.

Another study by (Stevenson, 2007) compared the proliferative rate of dermal fibroblasts derived from breast, abdominal and scalp skin using the ^{3}H-thymidine assay. In this study, mechanically wounding these cells also induced proliferation. In wounded dermal fibroblast cells, 17β-oestradiol increased cell proliferation, while having no effect on unwounded cells.

4.1.3 Aim

The previous chapter demonstrated that dermal fibroblasts from a similar anatomical region, derived from skin bearing either terminal (scalp) or vellus (facial) hair follicles exhibit different characteristics in terms of proliferation, morphology, and expression of IAPs; in particular XIAP. XIAP is an X-chromosome linked IAP, its expression is gender specific (Siegel et al., 2011). Ovariectomised mice treated with 17β-oestradiol resulted in an increase in XIAP levels in the endometrium (Leblanc et al., 2003). This suggests that IAPs potentially can be regulated by 17β-oestradiol, which could be an important pathway involved in improved wound healing by 17β-oestradiol.

Therefore, the first aim of this chapter was to compare the expression of ERα and ERβ *in situ*, in terminal and vellus hair bearing skin using immunofluorescent staining. A study of ERα and ERβ expression and localisation has been done in both males and females, showing no difference in expression (Thornton et al., 2003a). However, this is the first study to compare the expression of ERα and ERβ, between vellus and terminal hair bearing skin of the same donor.

The previous chapter also highlighted differences in the proliferative capability of matching cultures of DF(T) and DF(V) cells. Therefore, the second aim of this chapter was to determine whether DF(T) and DF(V) cells incubated with 17β-oestradiol exhibit different responses to proliferation and migration in the presence of 17β-oestradiol.

Since immunofluorescent staining is semi-quantitative and there was no reliable antibody for GPR30 at the time, the third aim was to quantitate the expression of mRNA for oestrogen receptors (ERα, ERβ and GPR30) in unwounded and mechanically wounded DF(T) and matching DF(V) cells using qRT-PCR. Since 17β-oestradiol can upregulate the expression of oestrogen receptors (Choi, 2007; Mahmoodzadeh et al., 2010), mRNA expression for oestrogen receptors was also analysed in unwounded and mechanically wounded DF(T) and DF(V) cells incubated in the presence of 17β-oestradiol.

4.2 Material and Methods

4.2.1 Human skin

Donor skin samples used in this chapter:

Donor ID	Age	Gender	S(T)	S(V)
OK18	56	Female	✓	✓
OK26	50	Female	✓	✓
OK27	57	Female	✓	✓

Table 4.1: Donor information for skin samples used for immunofluorescent staining

Matching vellus and terminal hair bearing skin samples from three donors were used for immunofluorescent staining, age range (50-57). S(T) – terminal hair bearing skin, S(V) – vellus hair bearing skin.

Donor ID	Age	Gender	DF(T)	DF(V)
OK14 [2, 3]	57	Female	✓	✓
OK15 [2, 3]	36	Female	✓	✓
OK17 [2]	53	Female	✓	✓
OK18 [1, 2, 3]	56	Female	✓	✓
OK21 [1]	64	Female	✓	✓
OK24 [1]	57	Female	✓	✓

Table 4.2: Donor information for samples used for dermal fibroblast cell culture

Donors used to culture matching dermal fibroblasts from terminal hair bearing skin DF(T), or vellus hair bearing skin (DF(V). [1] - used for cell migration, [2] - used for cell proliferation, [3] - used for PCR analysis.

4.2.2 Cryosectioning

Using the same method previously described in section 2.2.2, human skin sections from facelift patients containing terminal and vellus hair follicles (Table 4.1) were cut using a cryostat at 10µm thickness for immunofluorescent staining.

4.2.3 Immunofluorescent staining of human skin for ERα and ERβ

Using the same method previously described in section 2.2.4, immunofluorescent staining of skin samples for the expression of ERα and ERβ (Table 4.3) in matching terminal and vellus hair bearing skin was carried out.

Antibody (type):	Raised in:	Manufacturer:	Analysed in:	Dilution Ratio:
ERα (1˚)	R	SantaCruz	H	1:100
ERβ (1˚)	R	SantaCruz	H	1:100
FITC (2˚)	D anti R	Abcam	H	1:200

Table 4.3: Antibody dilution ratio used for immunofluorescence staining

A list of the primary and secondary antibodies used for immunofluorescent expression, and their dilutions. R – Rabbit, D – Donkey, H – Human.

4.2.4 Effect of 17β-oestradiol on dermal fibroblast cell proliferation *in vitro*

Matching DF(T) and DF(V) cultures from four donors at passage 3-4 (Table 4.2) were seeded at 10,000 cells per well in 6 well plates in phenol red free medium containing either 2% or 5% charcoal striped foetal bovine serum (Invitrogen) in the presence or absence of 1nM 17β-oestradiol (Sigma). Medium was changed every other day with cells counted every two days over a 12 day period; each treatment was done in triplicate wells, and each well was counted twice using a haemocytometer.

Activated carbon removes non-polar materials such as hormones and cytokines (Cao et al., 2009), allowing the study of oestrogen on dermal fibroblasts without additional hormone influences. In addition, since phenol red may act as a weak oestrogen (Berthois et al., 1986), all experimental procedures involving 17β-oestradiol were conducted in phenol red free medium, since phenol red increases migration in human scalp dermal fibroblast (Stevenson, 2007).

4.2.5 The effect of 17β-oestradiol on cultured dermal fibroblast migration in a scratch wound assay

To assess whether 17β-oestradiol had an effect on cell migration, a scratch wound assay, which is a well established method for assessing migration was carried out (see section3.2.3). Matching DF(T) and DF(V) cultures from three donors (Table 4.2) all at passage three, were seeded in 6 well plates at a density of 200,000 cells per well and left to grow until fully confluent (DF(V) – 4 days, DF(T) – 1 week). Growth medium was changed every three days. Once cells became fully confluent, the growth medium was removed, and the cells were washed twice with PBS. 2mls of serum free and phenol red free medium was added to each well and cells were incubated for a further 24 hours. Cells were scratched using the scratch wound device (Figure 3.2A). Cells were treated with different concentrations of 17β-oestradiol (1nM, 10nM and 100nM) and a vehicle control (0.001% absolute ethanol) in the presence of 10µg/ml mitomycin C as previously described (Stevenson et al., 2009). A template was placed under the 6 well plates (Figure 3.2B) and images were taken under a phase contrast microscope (Ernst Leitz Wetzlar GMBH, Germany) using a camera (Nikon coolpix4500 – 4x lens magnification, Japan) at time point zero and after 24 hours of incubation. Each condition was done in triplicate wells.

4.2.6 qRT-PCR

Primers were designed for ERα, ERβ and GPR30 using PrimerBlast (NCBI):

Primer:	Forward Sequence:	Reverse Sequence:	Annealing Temp:
ERα	TGGGCTTACTGACCAACCTG	CCTGATCATGGAGGGTCAAA	62.2˚C
ERβ	AGAGTCCCTGGTGTGAAGCAA	GACAGCGCAGAAGTGAGCATC	62.2˚C
GPR30	ATGGATGTGACTTCCCAAGC	GAACAGGCCGATCACGTACT	62.2˚C
GAPDH	TATAAATTGAGCCCGCAGCC	CGACCAAATCCGTTGACTCC	62.2 and 58.6˚C

Table 4.4: Primers used and their annealing temperature

Each primer was designed using PrimerBlast and annealing temperature optimised via quantitative PCR optimisation

Matching DF(T) and DF(V) cultures from three different donors (Table 4.2) at passage 3-4 were seeded into 6 well plates at a density of 200,000 cells per well and left to grow until fully confluent (DF(V) – 4 days, DF(T) – 1 week); growth medium was changed every three days. Once cells reached full confluence, the medium was removed and the cells were washed with PBS twice, before incubating in phenol red and serum free medium for 24 hours. Cells were either scratched in a "hash" (#) pattern using a 1ml pipette tip, or left unwounded. Cells were incubated with either 1nM 17β-oestradiol or vehicle control (0.001% absolute ethanol) for 24 hours, and then RNA extraction and quantitative real time PCR was carried out as described in section 3.2.5 using the primers shown in Table 4.4.

4.2.7 Statistics

For the cell migration assay to compare effects of different 17β-oestradiol concentrations on DF(T) and DF(V) cells, a one way analysis of variance (ANOVA) followed by a Dunnett's test, to determine significance between different concentrations and the control. For cell proliferation and PCR, an un-paired Students T-test of unequal variance was conducted between DF(T) and DF(V) cells.

4.3 Results

4.3.1 Expression levels and localisation of ERα and ERβ in terminal and vellus bearing skin

Immunofluorescent staining was carried out on matching terminal and hair bearing facial/scalp skin from three donors to compare the expression of ERα and ERβ (Figure 4.1).

4.3.1.1 A comparison of the expression of ERα between terminal and vellus hair bearing skin from the same donors

Overall, expression of ERα was lower in vellus hair bearing skin compared to terminal hair bearing skin (Figure 4.1A and C respectively), in both the epidermis and the dermis. In the vellus hair bearing skin, epidermal ERα expression was cytoplasmic, whilst in terminal hair bearing skin it was both cytoplasmic and nuclear.

The expression of ERα was not seen in the vellus hair follicle (Figure 4.1B). In contrast, in terminal hair follicles, ERα was highly expressed in the inner and outer root sheath, the dermal sheath, the epithelial cells of the hair matrix, with weak expression in the dermal papilla (Figure 4.1D and E).

A summary of the expression of ERα in terminal and vellus hair bearing skin is given in Table 4.5A.

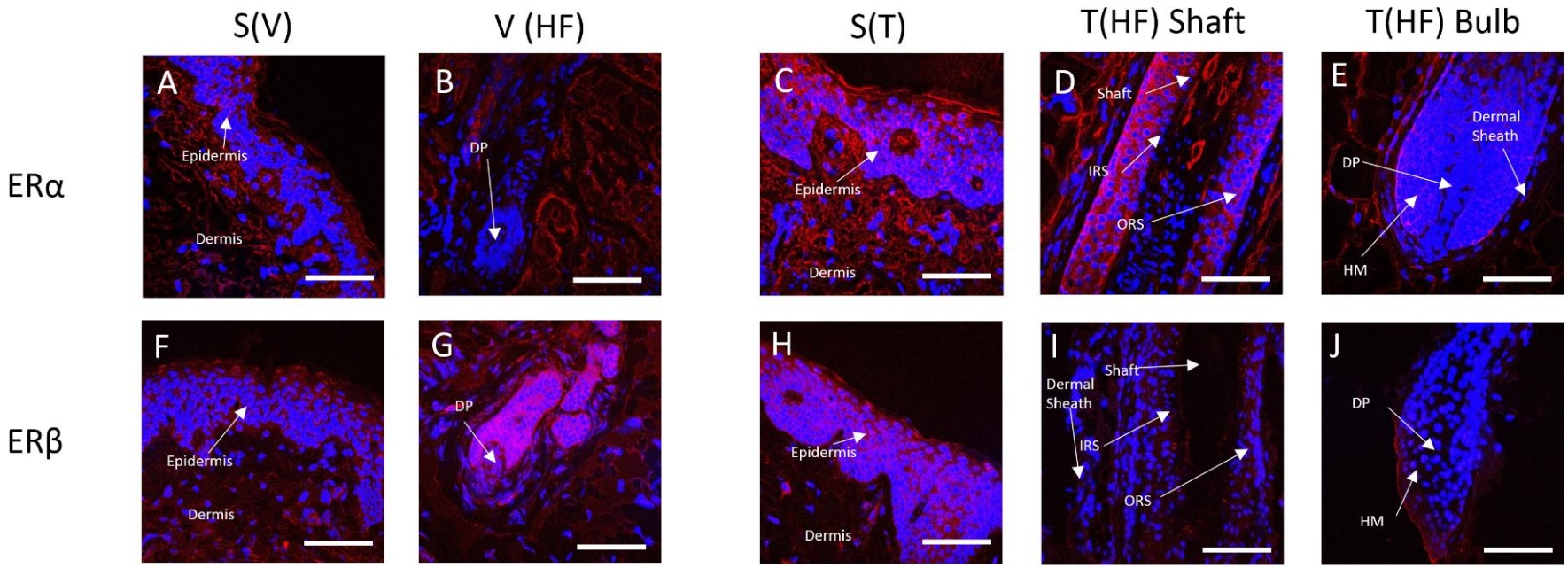


Figure 4.1: Expression of ERα and ERβ in human scalp containing terminal and vellus hair bearing skin and hair follicle

Oestrogen receptor (ER) immunofluorescent staining (red) with nuclear staining DAPI (blue) in the skin and hair follicle from vellus and terminal hair bearing skin. (A-E) Expression of ERα in female scalp skin. (F-J) Expression of ERβ in female scalp skin. (A and F), ER expression in vellus hair bearing facial skin (S(V)). (B and G) ER expression in vellus hair follicle (V(HF)) of vellus hair bearing facial skin. (C and H) ER expression in terminal hair bearing scalp skin (S(T)). (D and I) ER expression in the hair shaft region of terminal anagen hair follicles (T(HF)) in S(T). (E and J) ER expression of the bulb region of T(HF) in S(T).

DP – dermal papilla, HM – hair matrix, IRS – inner root sheath, ORS – outer root sheath. Magnification bar 100µm.

4.3.1.2 A comparison of the expression of ERβ between terminal and vellus hair bearing skin from the same donors

Epidermal expression of ERβ was lower in vellus hair bearing skin compared to terminal hair bearing skin (Figure 4.1A and C respectively); whilst in the dermis, expression of ERβ was low in both.

The expression of ERβ was low in the inner and outer root sheath and hair matrix, with little expression in the dermal papilla of the terminal hair follicles (Figure 4.1D and E). In comparison, higher expression was seen in vellus hair follicle compartments, including the inner and outer root sheath, hair matrix, dermal papilla and dermal sheath (Figure 4.1B). Expression of ERβ was cytoplasmic throughout.

A summary of the expression of ERβ in terminal and vellus hair bearing skin is given in Table 4.5A.

A	Skin		Hair Follicle				
ERα	EK	DF	IRS	ORS	HM	DP	DS
S(T)	+++	+++	+++	+++	+++	+	+++
S(V)	++	++	-	-	-	-	-

B	Skin		Hair Follicle				
ERβ	EK	DF	IRS	ORS	HM	DP	DS
S(T)	+++	+	+	+	+	-/+	+
S(V)	++	+	++	++	++	+	+

Table 4.5: Summary table for the expression and localisation of ERα and ERβ in terminal and vellus hair bearing skin

Expression of ERα and ERβ in terminal and vellus hair bearing skin and hair follicles. A) ERα expression in terminal and vellus hair bearing skin. B) ERβ expression in terminal and vellus hair bearing skin. ER – oestrogen receptor, EK – epidermal keratinocyte, DF – dermal fibroblasts, IRS – inner root sheath, ORS – outer root sheath, DP – dermal papilla, DS – dermal sheath, S(T) – terminal hair bearing skin, S(V) – vellus hair bearing skin. + low expression, ++ moderate expression, +++ high expression, n=3 donors. Red – 1 donor.

4.3.2 17β-oestradiol has no effect on DF(T) and DF(V) proliferation

DF(T) and matching DF(V) cells from four different donors were incubated in the presence or absence of 1nM 17β-oestradiol in the presence of either 2% or 5% charcoal stripped serum for 12 days and counted every other day (Figure 4.2).

In 2% charcoal stripped serum, DF(T) cells showed no increase in the rate of proliferation over a 12 day period in the presence or absence of 17β-oestradiol (Figure 4.2A). However, in DF(V) cells, in the absence of 17β-oestradiol cell number was significantly higher at day 2 ($p<0.0005$) and day 4 ($p<0.05$) only. Subsequent days showed no significant difference in cell number compared to day 0. No significant increase in cell number was observed in DF(V) cells in the presence of 17β-oestradiol (Figure 4.2A).

In 5% charcoal stripped serum, DF(T) cells showed no increase in number over a 12 day period in the presence or absence of 17β-oestradiol (Figure 4.2B). However, DF(V) cells showed a significant increase in the absence of 17β-oestradiol at days 2 ($p<0.05$), 4 ($p<0.005$), 6 ($p<0.05$), 8 ($p<0.05$), 10 ($p<0.005$) and 12 ($p<0.05$) and in the presence of 17β-oestradiol at day 2 ($p<0.005$), 4 ($p<0.005$), 6 ($p<0.05$), 8 ($p<0.005$), 10 ($p<0.005$) and 12 ($p<0.05$) compared to day 0 (Figure 4.2B). However the increased number of cells was not significantly different in DF(V) cells between cells incubated in the presence and absence of 17β-oestradiol.

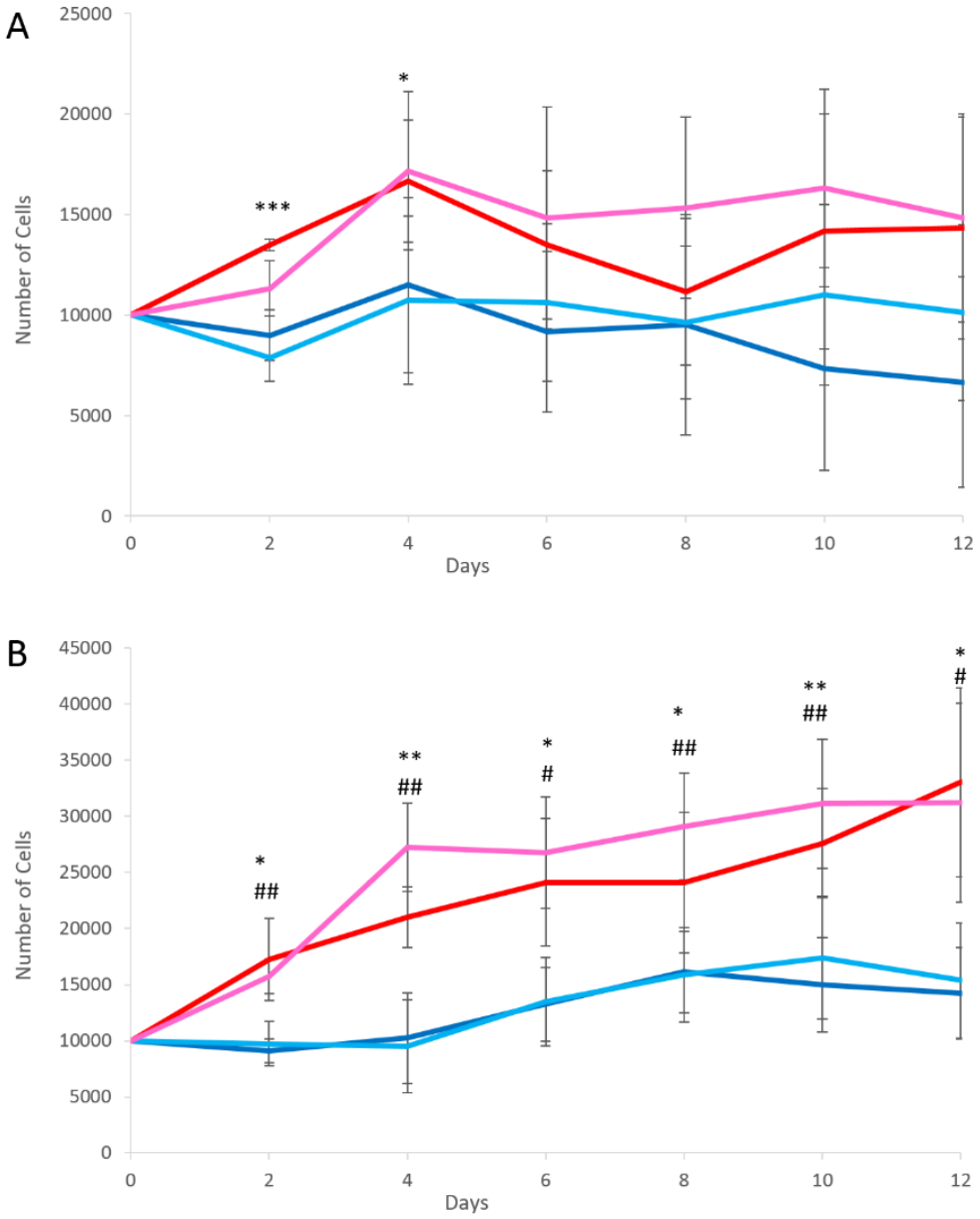


Figure 4.2: 17β-oestradiol has no effect on proliferation of DF(T) and matching DF(V) cells at 2 and 5% charcoal stripped serum

*Cell counting was used to determine the rate of proliferation over 12 days for DF(T) and matching DF(V) cells in the presence and absence of 17β-oestradiol in either 2% or 5% charcoal stripped serum. A) 2% charcoal stripped serum. B) 5% charcoal stripped serum. Dark blue – DF(T) vehicle control, light blue – DF(T) 1nM 17β-oestradiol, red – DF(V) vehicle control, pink – DF(V) 1nM 17β-oestradiol. n=4 donors, in triplicate wells, each well counted twice. $^{*/\#}$ p<0.05, $^{**/\#\#}$p<0.005, ***p<0.0005. $^{*/\#}$ - increase in DF(V) cell number in the absence/presence of 17β-oestradiol compared to day 0.*

4.3.3 17β-oestradiol accelerated migration of DF(T) but not DF(V) cultures in a scratch wound assay

The effect of 17β-oestradiol on migration was compared between matching DF(T) and DF(V) cells derived from three different female donors. Figure 4.3 gives the quantification of the mean cell migration 24 hours following scratching of matching DF(T) and DF(V) cultures derived from three donors. A significant increase in dermal fibroblast migration was seen in DF(T) cultures in the presence of 1nM 17β-oestradiol ($p<0.05$), and 100nM 17β-oestradiol ($p<0.005$); with distance migrated almost twice that of the dermal fibroblasts incubated with the vehicle control. In contrast, 17β-oestradiol did not significantly increase the migration of matching DF(V) cultures under the same conditions.

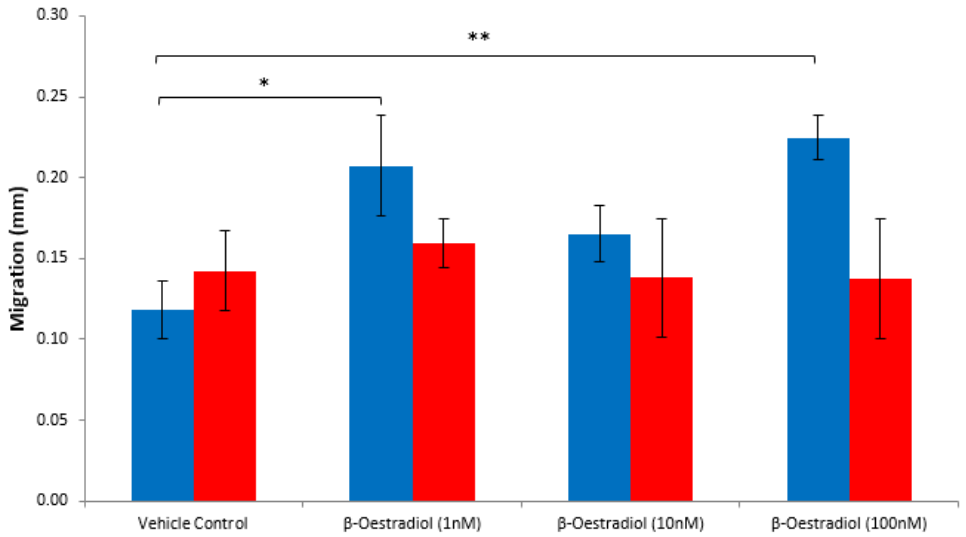


Figure 4.3: 17β-oestradiol increases migration of DF(T) but not DF(V) cells in a scratch wound assay

*Quantitative representation of cell migration after 24 hours was assessed in matching DF(T) and DF(V) cultures using a scratch wound assay in the presence of 17β-oestradiol at different concentrations (1nM, 10nM and 100nM) against vehicle control - 0.001% absolute ethanol (VC) all in the presence of 10µg/ml Mitomycin C. Red – DF(V) cells, blue – DF(T) cells. n=3 donors in triplicate wells at six different regional points. * p<0.05, ** p<0.005 (ANOVA – Dunnett's test).*

4.3.4 DF(T) cultures express higher mRNA levels of GPR30 and ERα than matching DF(V) cultures

Quantitative real time PCR was carried out on matching cultures of DF(T) and DF(V) derived from three female donors to compare the gene expression of the three oestrogen receptors, GPR30, ERα and ERβ in both unwounded and mechanically wounded dermal fibroblast cultures (Figure 4.4).

Expression of GPR30 was approximately three times higher in DF(T) cells compared to DF(V), ($p<0.05$). When DF(T) monolayers were mechanically wounded, mRNA expression of GPR30 was reduced to approximately half the level of expression seen in unwounded cells, ($p<0.05$). In contrast, no change in expression of GPR30 was seen when DF(V) cells were mechanically wounded (Figure 4.4).

The expression of mRNA for ERα was approximately three times higher in DF(T) cultures compared to matching DF(V) cultures, ($p<0.05$). When DF(T) cultures were mechanically wounded, mRNA expression of ERα was reduced to about 25% of its expression in unwounded cells, ($p<0.005$). In contrast there was no change in the expression of ERα in DF(V) cultures 24 hours after mechanical wounding (Figure 4.4).

A similar level of mRNA expression of ERβ was seen in matching cultures of DF(T) and DF(V) (Figure 4.4). However, mRNA expression in both DF(T) and DF(V) cultures was significantly reduced 24 hours following mechanical wounding, ($p<0.05$ and $p<0.0005$ respectively).

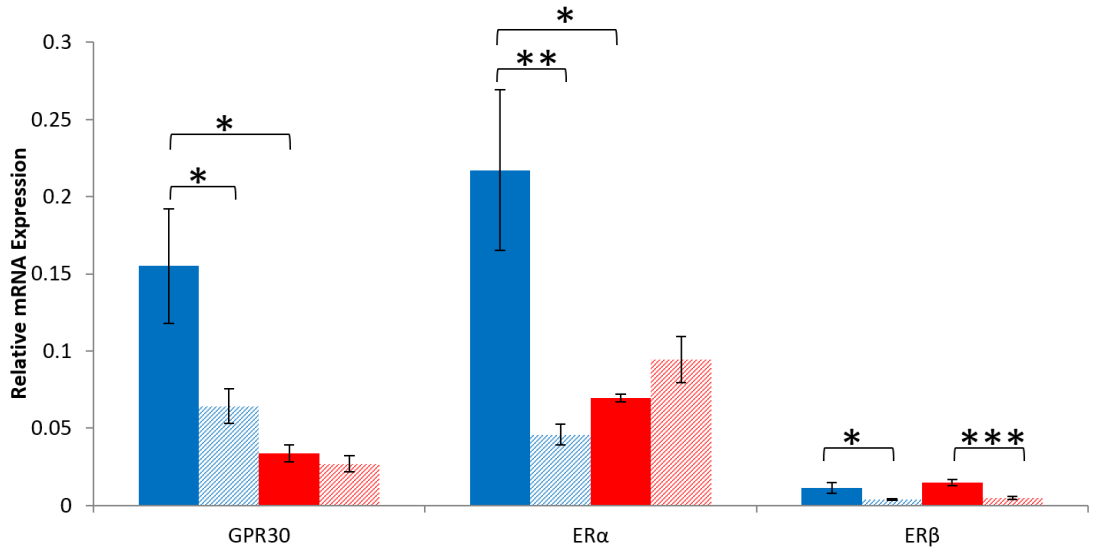


Figure 4.4: Higher levels of GPR30 and ERα in DF(T) cells compared to matching DF(V) cells, mRNA expression decreases in DF(T) cells when wounded

*A comparison of the mRNA expression of GPR30, ERα and ERβ in matching DF(T) and DF(V) cultures derived from female donors, (n=3) by qRT-PCR. Data was normalised against GAPDH under all conditions. Blue – DF(T), red – DF(V), solid – unwounded cells, hashed – wounded cells. Student T-test, n=3 donors in triplicates, * p<0.05 ** p<0.005, *** p<0.0005.*

4.3.5 Changes in GPR30, ERα and ERβ mRNA in matched DF(T) and DF(V) cultures in the presence of 17β-oestradiol following mechanical wounding

4.3.5.1 Increased mRNA levels of GPR30 in DF(V) cultures following mechanical wounding in the presence of 17β-oestradiol

17β-oestradiol had no effect on GPR30 mRNA expression of unwounded and mechanically wounded DF(T) monolayers (Figure 4.5A). Although 17β-oestradiol did not modulate the expression of GPR30 of unwounded donor matched DF(V) cultures, expression of GPR30 increased (~100%) when DF(V) monolayers were mechanically wounded ($p<0.05$).

4.3.5.2 Increased mRNA levels of ERα in matching DF(T) and DF(V) cultures following mechanical wounding in the presence of 17β-oestradiol

17β-oestradiol had no effect on mRNA expression of ERα of unwounded DF(T) cultures (Figure 4.5B). When DF(T) cells were mechanically wounded, 17β-oestradiol caused an increase (~50%) in ERα mRNA expression ($p<0.05$), but still only bringing it to a level significantly lower (~56%) than corresponding unwounded DF(T) cells.

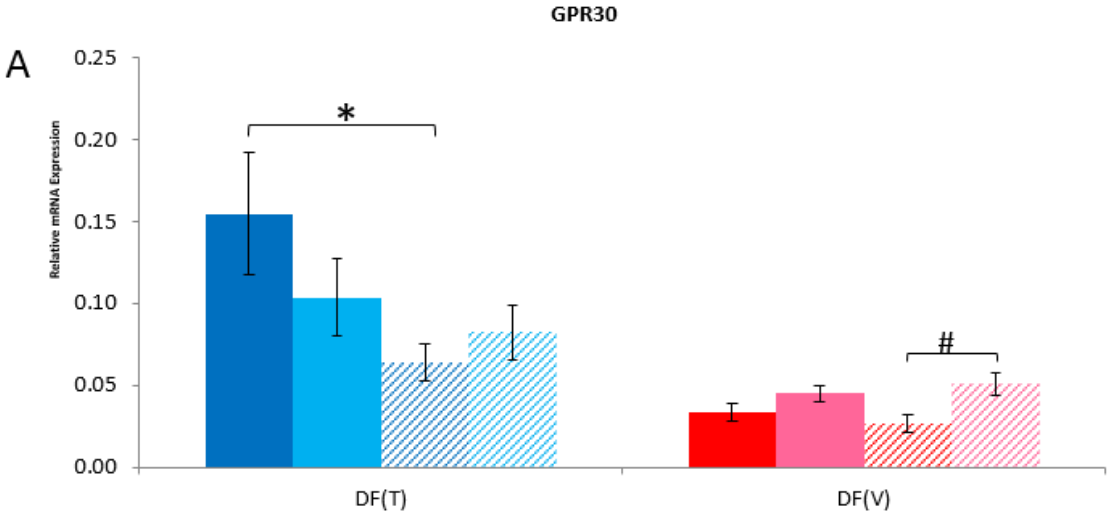
In matching unwounded DF(V) cultures, 17β-oestradiol had no effect on ERα mRNA levels (Figure 4.5B). There was an increase (~88%) in ERα mRNA levels in DF(V) cultures incubated with 17β-oestradiol following mechanical wounding ($p<0.05$), which was higher (~100%) than ERα expression in corresponding unwounded DF(V) cells ($p<0.05$).

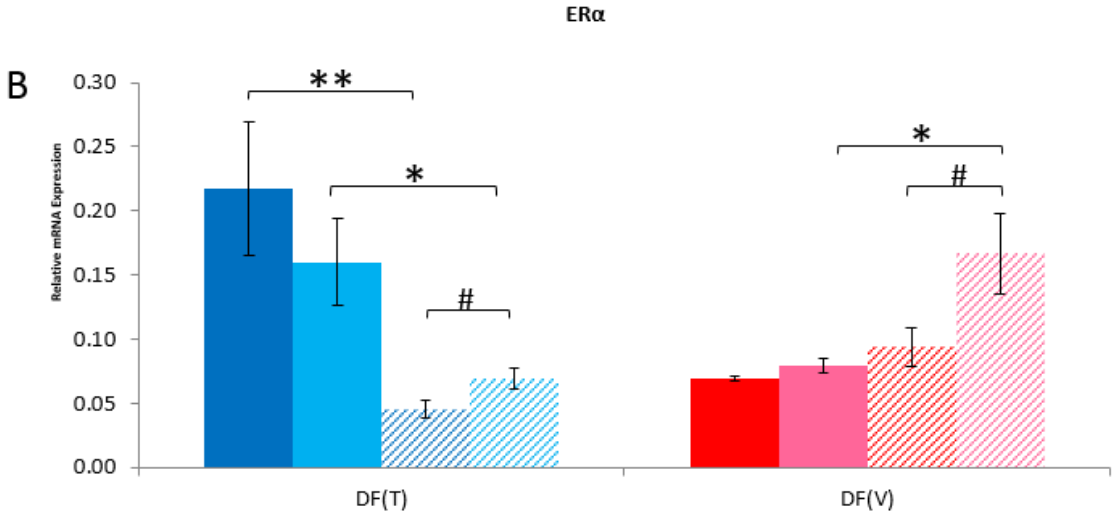
4.3.5.3 mRNA expression levels of ERβ were higher in wounded DF(T) cells in the presence of 17β-oestradiol

17β-oestradiol had no effect on ERβ mRNA expression in unwounded DF(T) cultures (Figure 4.5C). Expression of ERβ was increased (~133%) in

mechanically wounded DF(T) cells incubated with 17β-oestradiol, ($p<0.05$), but still only bringing it to a level significantly lower (~56%) than corresponding unwounded DF(T) cells.

In matching DF(V) cultures, mRNA expression of ERβ was not modulated with 17β-oestradiol treatment in either unwounded or mechanically wounded DF(V) cells. Therefore, mRNA expression in wounded, 17β-oestradiol treated cells was significantly lower (~33%) than corresponding unwounded cells ($p<0.005$).


GPR30
A
Relative mRNA Expression
0.25
0.20
0.15
0.10
0.05
0.00
*
#
DF(T)
DF(V)


ERα
B
Relative mRNA Expression
0.30
0.25
0.20
0.15
0.10
0.05
0.00
**
*
#
*
#
DF(T)
DF(V)

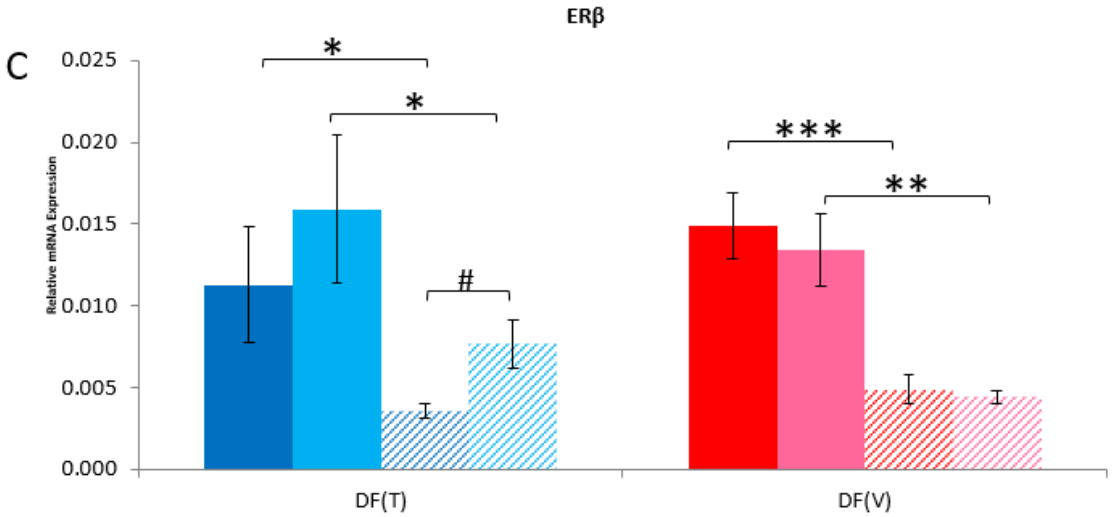

ERβ
C
Relative mRNA Expression
0.025
0.020
0.015
0.010
0.005
0.000
*
*
#
***
**
DF(T)
DF(V)

Figure 4.5: A comparison of mRNA expression levels for GPR30, ERα and ERβ in matching DF(T) and DF(V) cells in the presence of 17β-oestradiol in unwounded and wounded cells

*A comparison of the mRNA expression of GPR30, ERα and ERβ in matching DF(T) and DF(V) cultures derived from female donors treated with 17β-oestradiol, normalised against GAPDH using qRT-PCR. A) GPR30 mRNA expression in DF(T) and DF(V) cultures. B) ERα mRNA expression in DF(T) and DF(V) cultures. C) ERβ mRNA expression in DF(T) and DF(V) cultures. Dark blue – DF(T) vehicle control, light blue – DF(T) 1nM 17β-oestradiol, red – DF(V) vehicle control, pink – DF(V) 17β-oestradiol, solid bar – unwounded cells, hashed bar – wounded cells. n=3 donors in triplicates, Student T-test, */# p<0.05, ** p<0.005, *** p<0.0005. * - between unwounded and wounded cells, # - between wounded cells.*

4.4 Discussion

Previous studies have investigated the expression of ERα and ERβ in human skin and hair follicles of both sexes (Kwon et al., 2004; Thornton et al., 2003a, b). However, this is the first study to compare the expression of ERα and ERβ between terminal (scalp) and vellus (facial) hair bearing skin derived from the same donor.

4.4.1 *In situ* expression of ERα and ERβ in human terminal and vellus hair bearing skin

Expression of ERα in the epidermis and dermis of terminal hair bearing skin was slightly higher compared to matching vellus hair bearing skin (Figure 4.1 and Figure 2.9). This would suggest that scalp terminal hair bearing skin is more responsive and more regulated by 17β-oestradiol compared to corresponding facial vellus hair bearing skin from the same donor. It is important to note that the donor age range was 50-57, therefore their skin had been potentially sun-exposed for a long duration of time. Also, no information of topical medicinal cream application is known, which could potentially influence oestrogen receptor expression. A study by Rittie et al. (2008) compared the mRNA expression of oestrogen receptors ERα, ERβ and GPR30 in aged males and females (mean age 75) from three anatomical regions (sun protected hip, and photoaged forearm and face skin); the biopsies were taken from non matched donors, which could cause variability in data. Although mRNA expression for ERα, ERβ and GPR30 at the different anatomical regions was different, however it was not clear if expression levels varied due to light exposure or anatomical region. Their study showed a higher expression of ERα, ERβ and GPR30 in females in hip skin compared to sun-exposed forearm skin. However, mRNA expression for ERα and GPR30 appeared lower in forearm skin compared to face skin, while no differences were seen for ERβ. This pattern of expression was similar between males and females.

A study by Inoue et al. (2011) demonstrated that in both male and female human donors, (age range 10-79), there was no change in the expression of ERα with age. Anti-aging creams could potentially have an effect on oestrogen receptor expression. One ingredient that is used in anti-aging creams is epidermal growth factor (EGF). A study by Stoica et al. (2000) demonstrated that in MCF7 cell lines incubated with different concentrations of EGF, there was a reduction in ERα expression. Therefore, if the donors used anti-aging creams that contained EGF, it may explain why expression of ERα was reduced in vellus hair bearing skin compared to matching terminal hair bearing skin.

A similar expression pattern was seen for ERβ in the epidermis, with low expression in the dermis for both vellus and terminal hair bearing skin (Figure 4.1 and Figure 2.9). This could also be attributed to the possible topical applications applied. However, epidermal thickness in vellus hair bearing skin was significantly thinner than the epidermis from terminal hair bearing skin (Figure 2.7). Studies have shown that topical and oral application of oestrogen in castrated women increased epidermal thickness (Punnonen, 1971; Rauramo and Punnonen, 1969) (as reviewed by Brincat (2000)). Therefore, higher expression of oestrogen receptors (ERα and ERβ) in the epidermis of terminal hair bearing skin could be due to epidermal thickness.

17β-oestradiol in rats has been associated with inhibiting hair growth by repressing anagen (Oh and Smart, 1996), and in mice, it accelerated anagen development (Ohnemus et al., 2005). However, in humans, 17β-oestradiol has an opposite effect, where anagen is thought to be prolonged (Schumacher-Stock, 1981) (as reviewed by Ohnemus et al. (2006)). Terminal hair follicles expressed ERα however, vellus hair follicles did not (Figure 4.1). This could indicate that 17β-oestradiol is required for regulating human hair follicle cycling, if anagen is shortened and early onset of catagen occurs, it could prevent the hair follicle from becoming terminal hair follicles. In contrast to ERα, ERβ expression was lower in terminal hair follicles compared to vellus hair follicles. Even though in humans 17β-oestradiol has been shown to

prolong anagen (Schumacher-Stock, 1981), thereby controlling the hair follicle cycle, its effect on the hair cycle could be dependent on which receptor 17β-oestradiol activates. A study by Conrad et al. (2004) demonstrated using organ culture that hair follicle shaft elongation was evident as early as in the first day of 17β-oestradiol treatment. However, the effect of 17β-oestradiol via which receptor was not analysed. Each receptor could possibly control different aspects of the hair cycle, whether it is to prolong anagen or to decreases catagen and telogen. Unfortunately the effect of 17β-oestradiol on hair cycling is hard to analyse in humans, however, using similar techniques to Kondo et al. (1990) and Ohnemus et al. (2005), organ cultures can be used to treat hair follicles with either ERα or ERβ agonists and observe whether anagen is prolonged or whether hair follicles regress into catagen.

4.4.2 Effects of 17β-oestradiol on dermal fibroblast proliferation

A previous study by Stevenson (2007) compared the effect of 17β-oestradiol on the proliferation of human dermal fibroblasts derived from different anatomical regions using ^{3}H-thymidine uptake. Although fibroblasts derived from breast skin proliferated at a faster rate than those derived from scalp and abdominal skin, in the presence of 17β-oestradiol there was no change in the rate of proliferation in dermal fibroblasts derived from any of those regions.

A more recent study by Remoue et al. (2013) demonstrated that fibroblasts derived from abdominal skin from a young donor (23 years) (then repeated with two other donors of ages 21 and 27, with similar outcomes) treated with hormone levels circulating at menopause (with a reduction of 17β-oestradiol – see Table 4.6) caused cell proliferation to decrease, imitating aging conditions; in comparison to non menopausal hormone levels (with high concentration of 17β-oestradiol). This shows that 17β-oestradiol accelerates dermal fibroblast proliferation derived from abdominal skin.

The difference between the study conducted by Remoue et al. (2013) and that of Stevenson (2007), was that the age in which samples were obtained from. With aging, oestrogen receptor expression could be altered. The study by

Inoue et al. (2011) showed that ERβ expression decreased with age, which could influence cell proliferation if 17β-oestradiol acts on dermal fibroblasts through ERβ.

Hormones:	Non-menopausal Levels:	Menopausal Levels:
17β-oestradiol (pM)	750	60
Progesterone (nM)	60	6
Dehydroepiandrosterone (nM)	20	2
Growth Hormone (ng/ml)	5	1.5
IGF1 (ng/ml)	200	100

Table 4.6: Lower hormone levels in menopausal compared to non menopausal women

Hormone levels in the body of women who are non menopausal and menopausal (Remoue et al., 2013).

This cell counting study concurs with the ^{3}H-thymidine study by Stevenson (2007), and while DF(V) cultures proliferated at a faster rate than corresponding DF(T), 17β-oestradiol had no effect on proliferation over a 12 day period of either DF(T) nor DF(V) cells (Figure 4.2). The difference between this study and the study carried out by Stevenson et al. (2005), is that for cell counting over a 12 day period, serum had to be present. Serum concentrations had to be at low levels so DF(T) and DF(V) cells survived, and in order to remove any residual hormones, sex hormone binding globulin, and albumin binding proteins, charcoal stripped serum was used. This demonstrated that low serum concentration did not influence 17β-oestradiol in dermal fibroblast cell proliferation.

4.4.3 Effects of 17β-oestradiol on dermal fibroblast migration

Migration of DF(T) and DF(V) cells in the absence of 17β-oestradiol was similar (Figure 3.5), however in the presence of 17β-oestradiol, only DF(T) cells responded to 17β-oestradiol treatment, by increasing their rate of migration (Figure 4.3).

Expression of ERβ was similar in the dermis between terminal and vellus hair bearing skin. The expression of ERα was higher in the dermis of terminal hair

bearing skin compared to matching vellus hair bearing skin. This could indicate that ERα was responsible for the accelerated migration in DF(T) cells, therefore further studies were carried out to examine DF(T) and DF(V) oestrogen receptor expression and how 17β-oestradiol affects oestrogen receptor expression when wounded.

Stevenson (2007) demonstrated that abdominal and scalp human dermal fibroblasts migrated faster than dermal fibroblasts derived from breast skin, and that 17β-oestradiol accelerated migration of dermal fibroblasts derived from breast, abdominal and scalp skin. By using specific ERα and ERβ agonists, incubation with the specific ERα agonist accelerated the migration of abdominal dermal fibroblasts while the ERβ agonist had no effect (Stevenson et al., 2009), suggesting that stimulation of dermal fibroblast migration by 17β-oestradiol is modulated via ERα. To identify whether this is the case for DF(T) cells, ERα and ERβ agonists should be used to see if either are the cause for faster acceleration in DF(T) cells. Silencing different oestrogen receptor gene expression using siRNA could also help identify the pathway this involved. Also, the study by Stevenson (2007) demonstrated that migration of dermal fibroblasts derived from breast skin was significantly accelerated when incubated with 17β-oestradiol as early as after 4 hours, suggesting that the non-genomic pathway may be involved. Therefore GPR30 expression is important to study in both DF(T) and DF(V) cells.

4.4.4 Expression levels for oestrogen receptors in dermal fibroblasts

GPR30 is a cell surface receptor, which is required for the fast, non-genomic 17β-oestradiol signalling (Prossnitz et al., 2007). Therefore, quick responses to wound healing may act via GPR30. Antibodies for GPR30 at the time of studies were not available to confirm protein expression, however, since the protein levels of ERα and ERβ correlate with the mRNA levels (Figure 4.1/ Table 4.5 and Figure 4.4 respectively), it is likely that GPR30 mRNA expression may also reflect the protein levels.

GPR30 mRNA expression was higher in DF(T) cells compared to matching DF(V) cells, suggesting that DF(T) cells respond more quickly than DF(V) cells when stimulated with 17β-oestradiol. This pattern of expression was also seen for ERα, however, expression of ERβ was similar in DF(T) and DF(V) cells.

A study by Stevenson (2007) demonstrated that in dermal fibroblasts derived from breast skin, migration was accelerated with 17β-oestradiol at early time points (after 4 and 8 hours). This would suggest that the activity of 17β-oestradiol is through GPR30 and the non-genomic pathway, rather than the genomic pathway that involves ERα and ERβ. This could also explain why migration in DF(T) cells was more responsive to 17β-oestradiol, since GPR30 mRNA expression was higher in DF(T) cells compared to matching DF(V) cells. Since there are two potential pathways that could be involved, specific oestrogen receptor agonists and antagonists can be used to investigate which oestrogen receptor is involved. Silencing different oestrogen receptor gene expression by using siRNA in DF(T) and DF(V) cultures in a scratch wound assay and incubating them with 17β-oestradiol would help determine which receptor is involved in cell migration.

The mRNA expression of GPR30 and ERα was higher in DF(T) compared to matching DF(V) cells, while ERβ expression was similar between DF(T) and DF(V) cells (Figure 4.4). This coincides with the protein expression found in the dermis of terminal and vellus hair bearing skin (Figure 4.1 and Table 4.5). Higher expression of GPR30 in DF(T) cells would suggest that DF(T) cells react more quickly to 17β-oestradiol, as this involves the fast non-genomic pathway (Cheskis, 2004; Sharma and Prossnitz, 2011; Silva et al., 2010). DF(T) migration was accelerated with 17β-oestradiol treatment, however, migration was assessed after 24 hours, to investigate whether 17β-oestradiol has an earlier affect in accelerating cell migration, earlier time points need to be analysed, which would help establish if 17β-oestradiol is acting through GPR30.

Messenger RNA expression of oestrogen receptors was not altered in unwounded DF(T) and DF(V) cells when they were incubated with 17β-oestradiol. Suggesting that 17β-oestradiol only has an effect on damaged cells which could explain why 17β-oestradiol did not affect cell proliferation as the cells were not wounded. To measure and compare proliferation in unwounded and mechanically wounded cells in the presence and absence of 17β-oestradiol, a BrdU assay can be carried out on matching DF(T) and DF(V) cells from the same donor. There have been studies that have shown that 17β-oestradiol stimulates oestrogen receptor expression to sensitise cells to 17β-oestradiol (Zhou et al., 2001). However this study used mouse mesenchymal stem cells. Different species and different cells react differently to external stimulus. Another study by Detti et al. (2008) demonstrated that ERβ expression increased in abdominal fibroblasts when treated with 17β-oestradiol. The difference between the studies could be due to the anatomical region in which the fibroblasts were cultured from. Another study looking at human skin fibroblasts demonstrated that 17β-oestradiol upregulated ERβ as opposed to ERα (Haczynski et al., 2004). However, sample location was not indicated and therefore even though the skin samples were obtained from post menopausal women, different anatomical regions might influence 17β-oestradiol response.

When DF(T) cells were mechanically wounded, expression of all three oestrogen receptors decreased after 24 hours, only ERβ expression decreased in mechanically wounded DF(V) cells (Figure 4.4). Decreased expression of oestrogen receptor mRNA expression when mechanically wounded would suggest that cells need to be desensitised to 17β-oestradiol.

A study by Pomari et al. (2014) demonstrated that with mechanical wounding of dermal fibroblasts, activity of aromatase was reduced. Aromatase is the enzyme required for the conversion of testosterone to oestradiol and androstenedione to oestrone, increasing the local concentration of oestrogen. In this study by Pomari et al. (2014), the opposite effect was seen in epidermal keratinocytes that had been mechanically wounded, having increased

aromatase activity. Therefore it would be interesting to observe the effect of keratinocytes on dermal fibroblast population, and whether keratinocytes cultured from terminal and vellus hair bearing skin affects unwounded and wounded fibroblasts differently. The study by Pomari et al. (2014), looked at non-haired facial skin (mean age 50 years). When cells were mechanically wounded, aromatase activity was reduced therefore local oestradiol concentration was reduced. In the present study when DF(V) cells were mechanically wounded, only ERβ expression decreased with expression for GPR30 and ERα remaining the same. This could indicate that DF(V) cells when wounded are desensitised to 17β-oestradiol via ERβ. Also, the ratio of ERα: ERβ would be altered. The effect of 17β-oestradiol on cell transcription depends on the dimerization of the oestrogen receptors, either they homo- or hetro-dimerise to affect transcription (Li et al., 2004). Therefore, the impact on altering oestrogen receptor expression ratio could affect how 17β-oestradiol only accelerates migration in DF(T) but not DF(V) cells.

Interestingly, when wounded DF(T) cells were treated with 17β-oestradiol, ERα and ERβ mRNA expression increased, while in DF(V) cells, GPR30 and ERα increased compared to wounded non treated cells. Since migration was accelerated in DF(T) cells but not DF(V) cells, it would be assumed that this was via ERβ, as long as it leads to an increase in protein expression. Therefore, it would be important to measure protein expression in DF(T) and DF(V) cells when treated with 17β-oestradiol and see whether protein expression is also altered.

Expression of mRNA for ERα in both DF(T) and DF(V) cells increased in wounded cells treated with 17β-oestradiol, however, expression in DF(V) cells increased to higher levels compared to DF(T) cells. This would suggest that 17β-oestradiol has an effect on both DF(T) and DF(V) cells but more pronounced in DF(V) cells. There are many other factors that are involved in wound healing apart from migration and proliferation, such as collagen production and adhesion. A study by Stevenson et al. (2008a) demonstrated that in dermal fibroblasts derived from breast skin showed no difference in total

collagen secretion. However, in postmenopausal women there is a decrease in collagen content (Brincat et al., 1987; Thornton, 2002), and that with topical application of oestradiol, increased collagen fibres in the skin (Sauerbronn et al., 2000; Varila et al., 1995).

There are many different adhesion molecules that could be analysed such as integrins, cadherins and selectins. A study in mice osteoclasts showed that 17β-oestradiol reduced adhesion (Saintier et al., 2006), however, fibroblasts derived from different anatomical regions may react differently, a study by Shi-Wen et al. (1994) examined intracellular adhesion molecule 1 (ICAM1) expression in fibroblasts when treated with 17β-oestradiol. Expression did not increase in cells treated with 17β-oestradiol alone, however with the addition of TNFα, expression of ICAM1 increased. This could be due to the stress of the cells, therefore analysing adhesion molecules in unwounded and mechanically wounded condition in the presence and absence of 17β-oestradiol could help establish whether 17β-oestradiol plays a role in cell adhesion. Blocking different receptors by silencing the genes, or using specific oestrogen receptor agonists could help establish which receptor is involved.

When treated with 17β-oestradiol:	DF(V) cells compared to DF(T) cells
Proliferation	Higher
Migration	Slower

mRNA expression of:	DF(V) cells compared to DF(T) cells
GPR30	Lower
ERα	Lower
ERβ	Same

Effect of wounding cells on:	DF(V) cells compared to DF(T) cells
GPR30	Lower
ERα	Higher
ERβ	Same

Effect of 17β-oestradiol in unwounded cells on:	DF(V) cells compared to DF(T) cells
GPR30	Lower
ERα	Lower
ERβ	Same

Effect of 17β-oestradiol in wounded cells on:	DF(V) cells compared to DF(T) cells
GPR30	Same
ERα	Higher
ERβ	Lower

Table 4.7: mRNA expression levels of oestrogen receptors in DF(V) cells compared to matching DF(T) under different conditions

A comparison between DF(V) and DF(T) cells in proliferation and migration, and mRNA expression of oestrogen receptors GPR30, ERα, and ERβ under wounded and non-wounded conditions in the presence and absence of 17β-oestradiol.

4.5 Conclusion

This chapter has highlighted differences in the *in situ* expression of ERα and ERβ between terminal and vellus hair bearing skin from the same anatomical location of the same donor. In culture, while 17β-oestradiol did not modulate the proliferation of either DF(T) or DF(V), 17β-oestradiol accelerated the migration of DF(T) cells in a scratch wound assay.

Higher mRNA expression of oestrogen receptors GPR30 and ERα in DF(T) cells compared to matching DF(V) cells would suggest that DF(T) cells have quick responses to 17β-oestradiol (via GPR30), and that they are potentially more responsive compared to DF(V) cells.

Whilst incubation with 17β-oestradiol for 24 hours did not alter mRNA expression of any of the oestrogen receptors in unwounded DF(T) and DF(V) cells, when wounded, 17β-oestradiol increased the expression of ERα and ERβ in DF(T), and increased expression of GPR30 and ERα in DF(V) cells. Suggesting that accelerated migration in DF(T) 17β-oestradiol treated cells could potentially be via ERβ.

In summary, this chapter has demonstrated that 17β-oestradiol has different effects on matching DF(T) and DF(V) cells. Oestrogen receptor expression is also altered in unwounded and mechanically wounded conditions, indicating that 17β-oestradiol acts on DF(T) and DF(V) cells via different receptors to produce different responses.

Chapter 5

Inhibition of XIAP and the Effect of 17β-Oestradiol on DF(T) and DF(V) Cell Viability and IAP Expression

5 Inhibition of XIAP and the Effects of 17β-Oestradiol on DF(T) and DF(V) Cell Viability and IAP Expression

5.1 Introduction

From the previous chapter it was demonstrated that whilst 17β-oestradiol had no effect on dermal fibroblast proliferation, it accelerated DF(T) cell migration. Also the expression of oestrogen receptors was different between the two cell types suggesting that 17β-oestradiol has specific roles in each cell type. Expression of IAPs also vary between DF(T) and DF(V) cells, and a recent study by Fuchs et al. (2013) demonstrated the XIAP is essential in wound healing. Another study by Leblanc et al. (2003) demonstrated that in rat endometrium cell, 17β-oestradiol increased XIAP expression and decreased DIABLO. Therefore, regulation of IAPs in particular XIAP by oestrogen may be important in dermal fibroblasts during wound healing.

5.1.1 Importance of XIAP and other IAPs in wound healing

There are limited studies investigating the role of IAPs in wound healing, since most IAPs are generally associated with cancer cells. However, a recent study by Fuchs et al. (2013) demonstrated the importance of XIAP in wound healing. This study looked at single and double knockout of XIAP and its antagonist Sept4/ARTS in a murine model. In this study it was demonstrated that with the knockout of *Sept4/ARTS*, wound healing was accelerated, reepithelialisation was increased and hair follicle formation was seen 18 days post wound infliction, which was not seen in wild type mice. Also, the amount of TUNEL positive stem cells was decreased with *Sept4/ARTS* knockout. It is the protection of these stem cells that allowed the quick regeneration of the hair follicles and the epidermis. In this study, scar size was also compared between wild type and *Sept4/ARTS* knockout mice. Scar size significantly decreased with the absence of the XIAP antagonist. To further conclude that it is the function of XIAP that is essential to wound healing, *XIAP* knockout mice were also analysed. Wound healing was significantly impaired at day 5, 7, 9 and 11 compared to wild type. Also cleaved caspase 3 positive cells were

analysed. With *Sept4/ARTS* knockout mice, cleaved caspase 3 was significantly reduced, whilst with *XIAP* knockout, cleaved caspase 3 was significantly increased to wild type expression. This indicates the importance of XIAP in wound healing.

Another wound healing study investigating collagen contraction by Kobayashi et al. (2005), looked at collagen contraction by human foetal lung fibroblasts. It was shown in this *in vitro* study that while TGFβ1 stimulated collagen contraction, it also inhibited the expression of XIAP thereby stimulating apoptosis of the foetal lung fibroblast as detected by the TUNEL assay. This shows that there are other mechanisms in place that need to be taken into consideration during wound healing. IAPs are important in wound healing to also remove damaged cells and maintain homeostasis, they also remove redundant cells such as immune cells and senescent cells (Wang et al., 2009).

There are no other studies that have demonstrated the importance of IAPs in wound healing. In a murine model, XIAP was demonstrated to be important in wound healing. Therefore, XIAP and other IAPs deserve to be further studied to help establish how they can affect wound healing in human skin.

5.1.2 XIAP antagonist - embelin

Embelin, is a XIAP inhibitor, it functions by binding to the BIR3 domain (Nikolovska-Coleska et al., 2004); mimicking the action of DIABLO mimetics. In a recent study, embelin had an effect on proliferation, migration, cell apoptosis and XIAP expression of MCF7 cells (Sumalatha et al., 2014); cell proliferation and migration were reduced in the presence of embelin. Using the TUNEL assay, a higher number of MCF7 cells were positive for apoptosis with embelin treatment. XIAP mRNA expression was also reduced, following incubation with embelin; which indicates that embelin not only binds to XIAP to inhibit its function, but can also decrease XIAP transcription (Sumalatha et al., 2014). These results were compared to the effect of tamoxifen on ER positive breast cancer cell lines. Similar responses between embelin and tamoxifen were seen for both treatments in blocking cell migration and

reducing cell proliferation, suggesting that embelin may act as an anti-oestrogen (Sumalatha et al., 2014).

5.1.3 The effect of 17β-oestradiol on apoptosis and expression of IAPs

It has been suggested that oestrogens have a protective function in stressed cells (via oxidative stress) involved in skin wound healing in particular fibroblasts and keratinocytes by promoting the expression of anti-apoptotic proteins such as Bcl2 (Kanda and Watanabe, 2003; Yamaguchi et al., 2012).

In postmenopausal women, reduced oestrogen levels increase apoptosis in cells such as osteoblasts (Bradford et al., 2010). No studies have looked at the rate of apoptosis in human skin after menopause *in vivo*, nor how a decrease in oestrogen levels modulates apoptosis in keratinocytes and fibroblasts *in vitro*.

There have been limited studies looking at the effect of oestrogen on the Bcl-2 family and anti-apoptotic proteins in human skin. In cultured keratinocytes 17β-oestradiol prevents hydrogen peroxide induced apoptosis by increasing the expression of Bcl2 (Kanda and Watanabe, 2003). Using Western blotting and PCR, the expression of Bcl2 increased in human neonatal foreskin cultured keratinocytes treated with 17β-oestradiol.

In Jurkat (human T lymphocyte cell line), cIAP2 levels are under the control of NFκB. Using qRT-PCR, mRNA expression of cIAP2 in Jurkat cells that are responsive to NFκB induction by TNFα showed high expression of cIAP2, however, with Jurkat cells transfected with a truncated form of IκBα which lack the phosphoacceptor and ubiquitin attachment site, NFκB was not activated nor was cIAP2 expressed when cells were treated with TNFα (Chu et al., 1997). A study on human coronary artery endothelial cells (HCAEC) showed that 17β-oestradiol upregulated NFκB activation within 15 minutes of treatment (Hamilton et al., 2004). In this study, HCAEC cells were treated with 17β-oestradiol and ICI 182 780, which is an ERα and ERβ inhibitor, but an agonist

to the GPR30 receptor. Due to 17β-oestradiol's quick response, it would have been assumed that NFκB activation was through GPR30, however with ICI 182 780, NFκB was not activated, therefore, it was suggested that NFκB activation was via ERα, however, this was based on the admission of tamoxifen and reloxifen rather than potent oestrogen receptor specific agonists. Another study using rabbit corneal fibroblasts demonstrated that 17β-oestradiol decreased the phosphorylation of IκBα an inhibitor of NFκB. For NFκB to be activated, IκBα needs to be phosphorylated to dissociate from NFκB and activate it for translocation into the nucleus (Zhou et al., 2011). This shows that NFκB can be activated or repressed in different cell types in response to 17β-oestradiol. Since NFκB regulates IAP expression, in particular cIAP1, cIAP2 and XIAP (Wang et al., 1998; Wei et al., 2008), IAP regulation could potentially be through 17β-oestradiol via NFκB activation.

A study by Stanculescu et al. (2010) has shown that there is a link between the protective effects of 17β-oestradiol and IAPs. This study was conducted in MCF7 breast cancer cell lines, where DIABLO mimetics (cmpd3, MV1 and BV6) were used to block the effects of all IAPs and siRNA was used to silence XIAP, cIAP1 and cIAP2 before cell viability was studied using TNFα as a stress inducer for apoptosis. This study demonstrated that cIAP1 and cIAP2 were required for 17β-oestradiol to have a protective effect against TNFα. When cIAP1 and cIAP2 genes were silenced individually, cell viability was not restored when 17β-oestradiol was incubated with TNFα treated MCF7 cells. However, with the silencing of the XIAP gene, 17β-oestradiol was still able to protect cells. A combination of cIAP1 and cIAP2 silencing further disrupted the protective function of 17β-oestradiol. cIAP2 mRNA was upregulated by oestradiol in MCF7 breast cancer cells whilst cIAP1 and XIAP were not. This regulation was observed in cells positive for oestrogen receptors (mainly ERα) (Stanculescu et al., 2010).

A study by Yin et al. (2008) showed using qRT-PCR that in murine uterine epithelial cells 17β-oestradiol induced the expression of NAIP via ERα thereby protecting uterine epithelial cells from apoptosis. Furthermore, NAIP was

required for the protective effect of oestradiol since knockout of the *Naip* gene resulted in higher levels of apoptotic cells detected by TUNEL even in the presence of oestradiol, with a higher level of caspase 3 and caspase 9 activation (Yin et al., 2008).

XIAP has been associated with elevated cell proliferation and VEGF production in rat Muller cells (glial cells of the retina), since inhibiting XIAP using embelin (an XIAP inhibitor) caused a reduction in VEGF secretion, indicating that XIAP is important in regulating VEGF secretion (Sun et al., 2013).

Due to the recent study by Fuchs et al. (2013), it has been established that XIAP is an important IAP in murine wound healing. This protein is gender specific, a study in male and age related female murine brain showed higher expression of XIAP in females than in corresponding males, whilst ovariectomy did not alter XIAP expression in females (Siegel et al., 2011). In ovariectomised rats, a three day treatment of 17β-oestradiol caused an increase in XIAP expression and decrease DIABLO in the endometrium, indicating that XIAP is regulated by 17β-oestradiol, this could potentially be via the down regulation of DIABLO expression (Leblanc et al., 2003).

5.1.4 Effects of 17β-oestradiol on caspase activity

In osteoblasts, the protective effects of oestrogen have been attributed to the reduction of caspase 3 and 7 activity, since TNFα induced apoptosis by activating caspase 3, and treatment with 17β-oestradiol reduced caspase 3 activity (Bradford et al., 2010). The protective function of 17β-oestradiol in bovine aortic endothelial cells following TNFα induced apoptosis was demonstrated using a cell viability assay (Koga et al., 2004). In this study, the percentage of cells undergoing apoptosis was determined when cells were treated with TNFα in the absence and presence of 17β-oestradiol. 17β-oestradiol caused a reduction in the percentage of cells undergoing apoptosis. A study by Li et al. (2011) demonstrated using Western blotting and qRT-PCR, that in ovariectomised rats, 17β-oestradiol protects against cell death by

preventing cleavage of caspase 3 in the brain. This is achieved through ERα, since when ERα was blocked using the antagonist MPP, the protective effect of 17β-oestradiol was lost; this was not seen when an ERβ antagonist was used (Li et al., 2011). To date, there are no studies analysing the effects of 17β-oestradiol on caspase activity in skin and hair follicles.

5.1.5 Aim

The previous chapter, demonstrated that 17β-oestradiol did not affect proliferation of DF(T) and DF(V) cells. However, DF(T) cell migration was accelerated in response to 17β-oestradiol while corresponding DF(V) cells did not. Due to the changes of oestrogen receptor mRNA expression in wounded DF(T) and DF(V) cells, accelerated migration in DF(T) cells incubated with 17β-oestradiol could potentially be via ERβ. Expression of oestrogen receptors did not change with 17β-oestradiol treatment in unwounded cells, however when mechanically wounded, expression of ERα and ERβ increased in DF(T) cells while ERα and GPR30 increased in DF(V) cells.

The first aim of this chapter was to establish whether 17β-oestradiol has a protective effect on cell viability of DF(T) and DF(V) cells when XIAP expression is blocked by embelin in both unwounded and mechanically wounded DF(T) and DF(V) cells.

Since caspase 3 is the effector caspase, it is an important caspase to become activated when apoptosis occurs, therefore the second aim of this chapter was to determine whether caspase 3 activity was increased when XIAP was inhibited by embelin and whether the response of 17β-oestradiol can counteract changes in caspase 3 activity.

Finally, to establish whether 17β-oestradiol can regulate the mRNA expression of XIAP, NAIP, cIAP2 and Apollon or their antagonists DIABLO and Xaf1 in either DF(T) or DF(V) cells, and whether this changes when cells are mechanically wounded.

5.2 Materials and Methods

5.2.1 Human tissue samples used

Donor samples used in this chapter:

Donor ID	Age	Gender	DF(T)	DF(V)
OK14 [1, 3]	57	Female	✓	✓
OK15 [1, 3]	36	Female	✓	✓
OK17 [1, 2]	53	Female	✓	✓
OK18 [2, 3]	56	Female	✓	✓
OK23 [2]	51	Female	✓	✓

Table 5.1: Donor information for skin samples used for immunofluorescent staining

Donor samples used to culture matching dermal fibroblasts from either terminal hair bearing skin DF(T), or from vellus hair bearing skin (DF(V). [1] - cell viability, [2] - ELISA, [3] - PCR analysis.

5.2.2 Cell viability of matching DF(T) and DF(V) in the presence of embelin (XIAP inhibitor) and 17β-oestradiol

Matching DF(T) and DF(V) cells cultured from three female donors at the same passage (P4) were seeded in 24 well plates in growth medium (DMEM with 15% FBS) at a cell density of 10,000 cells per well and left to grow to confluence (DF(V) – ~5 days, DF(T) – ~1 week); with a medium change every 3 days. Once fully confluent, cells were washed with PBS twice and incubated in serum free and phenol red free DMEM for 24 hours. Wells were separated into groups, for non wounded conditions and wounded conditions; wounded wells were scratched using a 10µl pipette tip in a hash (#) pattern. The growth medium was removed, and cells were incubated in serum free, phenol red free medium supplemented with either vehicle control (0.1% DMSO), 17β-oestradiol (10nM), embelin (Sigma) (50µM), and embelin (50µM) and 17β-oestradiol (10nM) together; unwounded and wounded cells were assessed in triplicate wells.

After 24 hours, medium was removed carefully so as to minimise dislodging cells that might be on the verge of detaching. Without washing, 100µl of 0.05% trypsin/EDTA was added to the wells; once cells started to condense, they were placed in an incubator for 5 minutes. The 24 well plates were then disturbed by knocking on the sides to dislodge cells, once all cells were dislodged, 200µl of growth medium was added to each well, bringing the total volume to 300µl. 15µl of the cell suspension from each individual well was added to 15µl of filtered 0.4% trypan blue solution in an Eppendorf. Using a haemocytometer, each cell suspension was counted twice to determine the number of viable and non viable cells and cell viability determined under the different experimental conditions.

5.2.3 Caspase 3 activation in matching DF(T) and DF(V) cells using ELISA

Using the Caspase-Glo 3/7 Assay kit (Promega), caspase activity was measured in matching cultures of DF(T) and DF(V) derived from three donors at the same passage number (P4). Cells were seeded in 96 well plates at 5,000 cells per well in growth medium (DMEM with 15% FBS) and left to grow until fully confluent (DF(T) – 3 days, DF(V) cells – 4 days). Cells were washed with PBS twice and incubated in serum free and phenol red free DMEM for 24hours. The cells were either scratched using a 10µl pipette tip in a plus (+) pattern or left unwounded. The medium was removed and duplicate wells incubated with either vehicle control (0.4% DMSO), 17β-oestradiol (10nM), embelin (50µM), and embelin (50µM) + 17β-oestradiol (10nM) and incubated for 24hours. The caspase 3 assay kit reagent was added at a 1:1 ratio (assay:medium) to the cells and the plates were left on a shaker wrapped in foil for 1hour. Using a luminescent plate reader (Tecan, infinite M200), caspase 3 activity was measured.

5.2.4 PCR analysis of IAPs on 17β-oestradiol treated DF(T) and DF(V) cells in unwounded and mechanical wounded conditions

Using the same principles in methodology for qRT-PCR described in section 3.2.5; matching DF(T) and DF(V) cells (passage 3-4) were cultured in 6 well plates and when fully confluent washed then incubated in serum free and phenol red free medium for 24 hours. Half the wells were mechanically wounded by scratching a hash (#) pattern using a 1ml pipette tip; cells were either incubated with vehicle control (absolute ethanol 0.001%) or 17β-oestradiol (1nM) for 24 hours. Using the same methodology described in section 3.2.5, RNA was extracted and cDNA was synthesised. Expression of IAPs was normalised against GAPDH.

5.2.5 Statistics

For the cell viability, caspase 3 activity and qRT-PCR, Student T-test was carried out between DF(T) and DF(V) cells.

5.3 Results

5.3.1 Embelin decreased cell viability in matching DF(T) and DF(V), which was partially restored in the presence of 17β-oestradiol

Cell viability was assessed using the trypan blue exclusion dye on matching DF(T) and DF(V) cultures, in the presence of embelin, a potent XIAP inhibitor, or 17β-oestradiol, or embelin and 17β-oestradiol together (Figure 5.1).

In matching DF(T) and DF(V) cultures, cell viability under control conditions was around 98% (Figure 5.1A and B respectively). Mechanical wounding caused no change in cell viability in either DF(T) or DF(V) cultures. Incubation with 17β-oestradiol did not alter cell viability of DF(T) and DF(V) cells when mechanically wounded or left unwounded (Figure 5.1A and B respectively).

However, with embelin treatment, cell viability was significantly reduced in unwounded DF(T) cultures to 77% ($p<0.005$), and to 74% ($p<0.005$) in mechanicaly wounded cells (Figure 5.1A). In corresponding DF(V) cultures, embelin decreased cell viability in unwounded and mechanically wounded cells to 85% ($P<0.0005$) and 87% ($P<0.05$) respectively (Figure 5.1B).

In DF(T) cultures, the presence of 17β-oestradiol in embelin treated cells resulted in a partial, but significant increase in cell viability, with cell viability increasing to 88% in unwounded and 87% in mechanically wounded DF(T) cultures ($p<0.05$) (Figure 5.1A). In matching unwounded DF(V) cultures, 17β-oestradiol in embelin treated cells resulted in a partial, but significant increase in cell viability to 93% ($p<0.05$) (Figure 5.1B). In mechanically wounded cells, the presence of 17β-oestradiol in embelin treated cells did not significantly change cell viability.

When comparing the effect of embelin on cell viability between matching DF(T) and DF(V) cultures, embelin induced a significantly higher reduction in cell viability in DF(T) cultures compared to DF(V) cells, in both unwounded ($p<0.05$) and mechanically wounded cells ($p<0.05$).

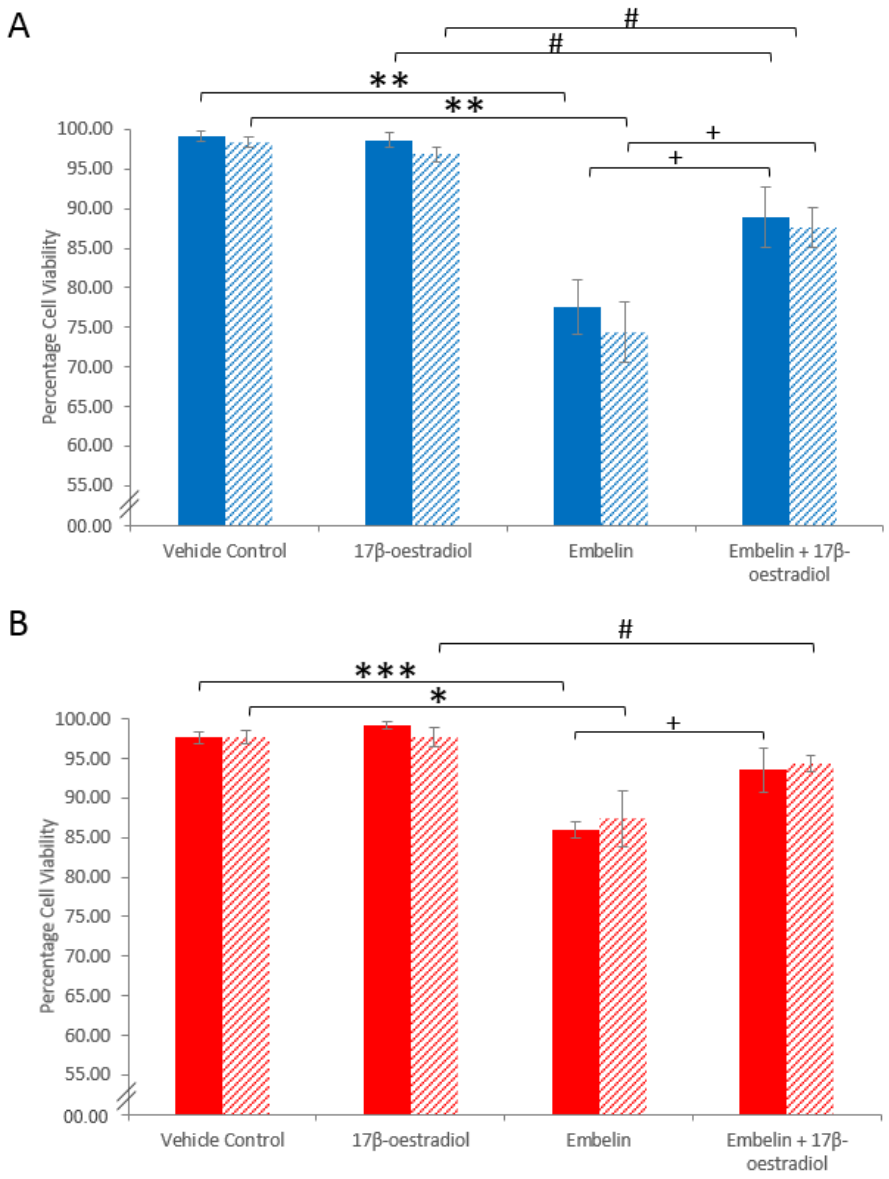


Figure 5.1: Cell viability was reduced in DF(T) and DF(V) cells when treated with embelin and restored with the addition of 17β-oestradiol

*Cell viability in matching DF(T) and DF(V) cells treated with Embelin and 17β-oestradiol. A) DF(T) cell viability. B) DF(V) cell viability. Blue – DF(T) cells, red – DF(V) cells, solid bar – unwounded cells, hashed bar – mechanically wounded cells. n=3 donors, in triplicate wells counted in duplicates. */#/+p<0.05, **p<0.005, ***p<0.0005. (Students T-test). * - between vehicle control and embelin, # - between 17β-oestradiol and embelin and 17β-oestradiol together, + - between embelin and embelin and 17β-oestradiol together.*

5.3.2 Embelin does not alter the activity of caspase 3 in unwounded and mechanically wounded dermal fibroblasts

Since the XIAP antagonist embelin decreased viability of DF(T) and DF(V) cells and this was partially counteracted by 17β-oestradiol, changes in the activity of caspase 3 was measured when unwounded and mechanically wounded dermal fibroblasts were incubated in the presence and absence of embelin with, or without, 17β-oestradiol (Figure 5.2).

Neither embelin alone nor 17β-oestradiol alone significantly altered the activity of caspase 3 in matching DF(T) and DF(V) cultures, whether they were unwounded or mechanically wounded (Figure 5.2A and B respectively).

The addition of 17β-oestradiol to embelin treated DF(T) cells significantly reduced caspase 3 activity compared to embelin treatment alone ($p<0.05$) in both unwounded and mechanically wounded cells (Figure 5.2A). In contrast, in matching DF(V) cultures, 17β-oestradiol did not significantly reduce caspase 3 activity in the presence of embelin in either unwounded or mechanically wounded cells (Figure 5.2B).

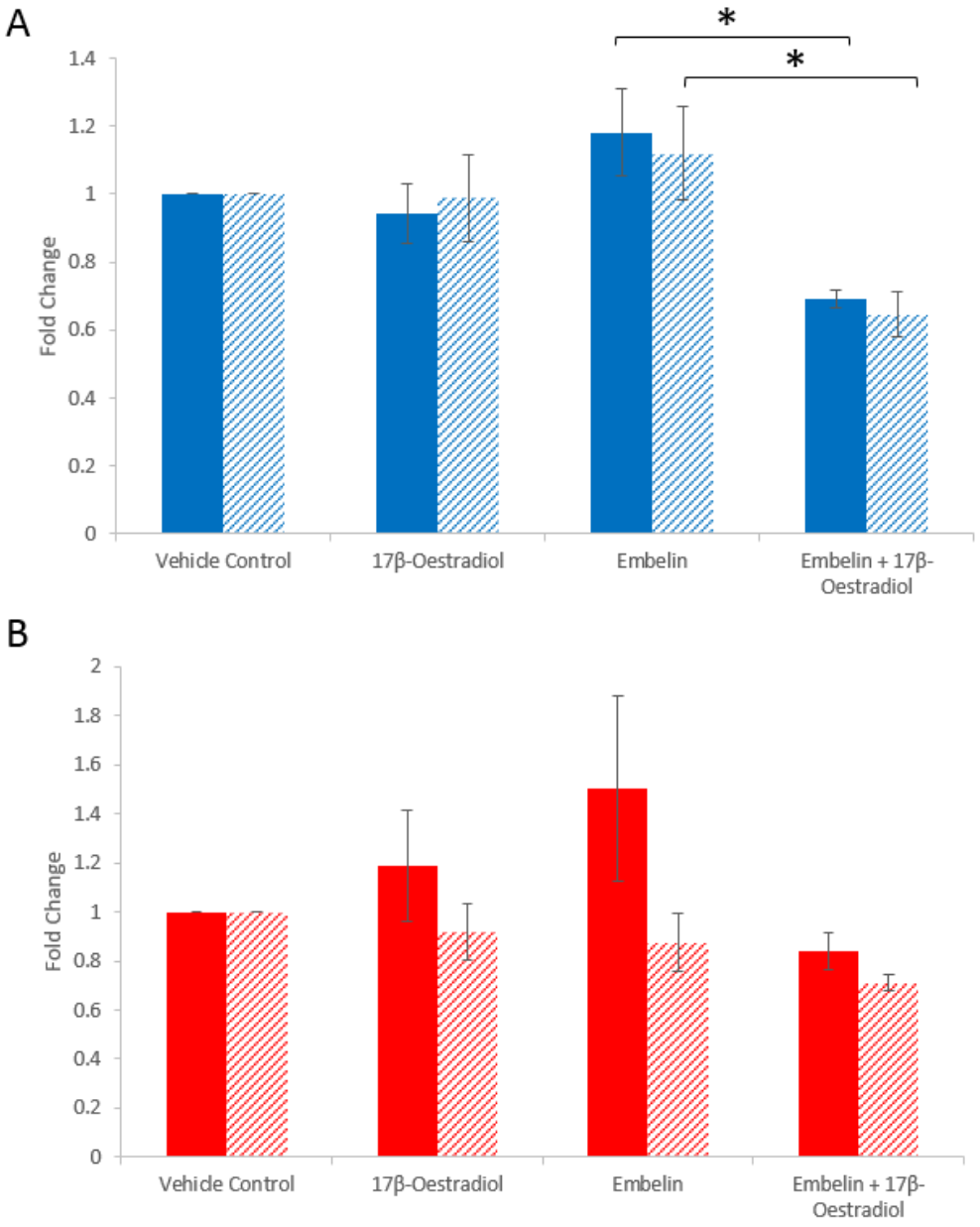


Figure 5.2: Embelin causes no change in caspase 3 activity but a reduction when co-treated with 17β-oestradiol *in vitro*

*Caspase 3 activity measured by ELISA in matching DF(T) and DF(V) cells incubated with vehicle control (DMSO 0.4%), 17β-oestradiol (10nM), embelin (50μM), embelin and caspase 3 inhibitor (10 μM), and embelin and 17β-oestradiol. A) Caspase 3 activity in DF(T) cells. B) Caspase 3 activity in DF(V) cells. Blue – DF(T) cells, red – DF(V) cells, solid bar – unwounded cells, hashed bar – mechanically wounded cells. n=3 donors in duplicates. *p<0.05, (Students T-test).*

5.3.3 Does 17β-oestradiol modulate mRNA expression of IAPs and IAP antagonists in DF(T) and DF(V) cells following mechanical wounding *in vitro*?

Figure 5.3 represents qRT-PCR data on IAP mRNA expression in matching DF(T) and DF(V) cells in the presence and absence of 17β-oestradiol in both unwounded and mechanically wounded cells.

5.3.3.1 17β-oestradiol increased XIAP mRNA expression in unwounded and mechanically wounded DF(T) and unwounded DF(V) cells, but decreased expression in mechanically wounded DF(V) cells

17β-oestradiol increased the mRNA expression of XIAP by ~24% in unwounded DF(T) cells ($p<0.05$), but increased XIAP mRNA expression in mechanically wounded cells by ~68% ($p<0.0005$) (Figure 5.3A). In corresponding DF(V) cells, expression of XIAP was increased in the presence of 17β-oestradiol in unwounded cells by ~15% ($p<0.05$). Whilst, it was reduced by ~30%, in wounded cells incubated with 17β-oestradiol ($p<0.0005$) to levels significantly lower (~33%) than the corresponding unwounded cells $p<0.0005$).

5.3.3.2 17β-oestradiol increased Apollon mRNA expression in DF(T) and decreased expression DF(V) in unwounded and mechanically wounded cultures

17β-oestradiol increased the expression of Apollon in both unwounded by ~60%, and mechanically wounded by ~125% DF(T) cells ($p<0.05$ and $p<0.0005$ respectively). In contrast, in corresponding DF(V) cells incubated with 17β-oestradiol for 24 hours, mRNA expression of Apollon decreased in both unwounded cells by ~57% and wounded cells by ~57% ($p<0.05$ and $p<0.005$ respectively). The decrease in expression in mechanically wounded cells by 17β-oestradiol, reduced expression to levels significantly lower (~50%) than the corresponding unwounded cells, $p<0.005$ (Figure 5.3B).

5.3.3.3 17β-oestradiol increased cIAP2 mRNA expression in mechanically wounded DF(T) and decreased expression in mechanically wounded DF(V) cultures

The expression of cIAP2 in unwounded DF(T) cells was not altered in the presence of 17β-oestradiol, but in mechanically wounded DF(T) cells, it was increased by ~200% ($p<0.0005$) (Figure 5.3C). In contrast, 17β-oestradiol reduced cIAP2 expression in unwounded DF(V) cells by ~85% ($p<0.0005$), but did not alter the expression in mechanically wounded cells.

5.3.3.4 17β-oestradiol increased NAIP mRNA expression in unwounded and mechanically wounded DF(T) cells, but did not alter expression in DF(V) cells

In unwounded DF(T) cells, NAIP mRNA expression was increased in the presence of 17β-oestradiol by ~83% ($p<0.0005$), while in mechanically wounded cells, it was increased by ~433% ($p<0.0005$), (Figure 5.3C), bringing it to a level significantly higher (~191%) than the corresponding unwounded cells ($p<0.005$). In corresponding DF(V) cells, 17β-oestradiol did not alter NAIP expression in either unwounded or mechanically wounded cells. However, the level of expression of NAIP in mechanically wounded DF(V) was significantly lower (~37%) than that of unwounded cells in the presence of 17β-oestradiol ($p<0.0005$).

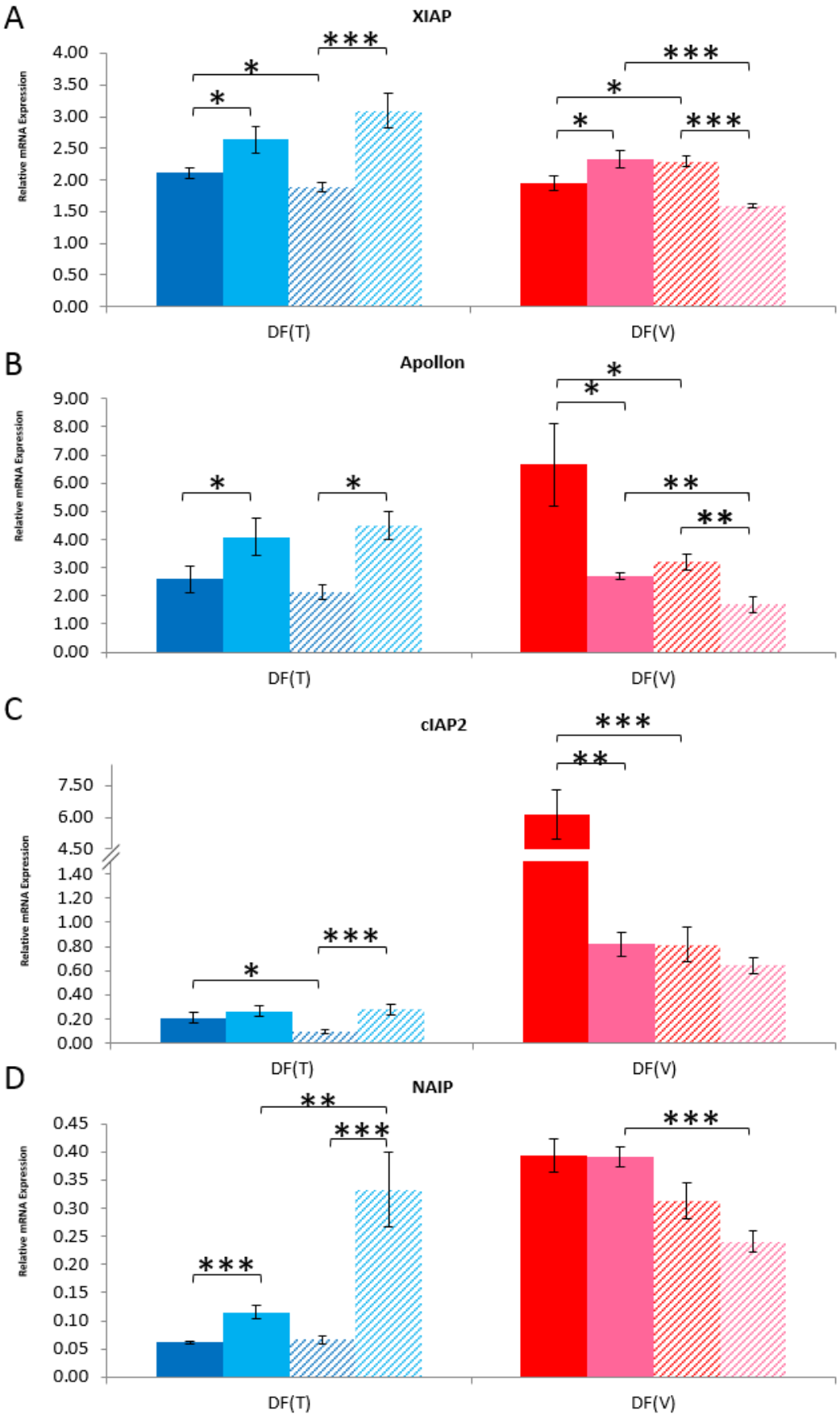

A
XIAP
Relative mRNA Expression
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
DF(T)
DF(V)
B
Apollon
Relative mRNA Expression
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
DF(T)
DF(V)
C
cIAP2
Relative mRNA Expression
7.50
6.00
4.50
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
DF(T)
DF(V)
D
NAIP
Relative mRNA Expression
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
DF(T)
DF(V)

Figure 5.3: Increased expression of IAPs in DF(T) cells with 17β-oestradiol in unwounded and mechanically wounded cells *in vitro*, with decreased expression in DF(V) cells

*IAP transcription expression analysed via qRT-PCR for matching DF(T) and DF(V) cells in unwounded and mechanically wounded conditions, in the presence and absence of 17β-oestradiol normalised against GAPDH. A) mRNA expression for XIAP. B) mRNA expression for Apollon. C) mRNA expression for cIAP2. D) mRNA expression for NAIP. Dark blue – vehicle control DF(T), light blue – 17β-oestradiol treated DF(T), red – vehicle control DF(V), pink – 17β-oestradiol treated DF(V), solid bar – unwounded cells, hashed bar – wounded cells. n=3 donors. Students T-test, * p<0.05, ** p<0.005, *** p<0.0005.*

5.3.3.5 17β-oestradiol increased mRNA expression of DIABLO in matching DF(T) and DF(V) under wounded and non-wounded conditions

When unwounded DF(T) cultures were incubated with 17β-oestradiol for 24 hours, expression of DIABLO mRNA increased by ~50%, $p<0.05$; however, in wounded cells, mRNA expression of DIABLO increased by ~500% ($p<0.0005$) to levels significantly higher (~100%) than corresponding unwounded cells ($p<0.005$) (Figure 5.4A). In corresponding DF(V) cells, 17β-oestradiol increased DIABLO expression by ~133% in unwounded cells ($p<0.0005$), and also increased DIABLO mRNA expression by ~300% in mechanically wounded cells ($p<0.0005$) (Figure 5.4A).

5.3.3.6 17β-oestradiol increased mRNA expression of Xaf1 in unwounded and mechanically wounded DF(T) cells and decreased expression in mechanically wounded DF(V) cells

In a similar manner to DIABLO, 17β-oestradiol increased Xaf1 mRNA expression in unwounded DF(T) cells by ~70% ($p<0.05$), and by 266% in mechanically wounded cells ($p<0.005$) to levels significantly higher (89%) than the corresponding unwounded cultures ($p<0.05$) (Figure 5.4B). In contrast, in matching DF(V) cells, while 17β-oestradiol had no effect on Xaf1 mRNA expression in unwounded cells, it decreased expression in mechanically wounded cells by ~53% ($p<0.0005$), to levels significantly lower (~72%) than corresponding unwounded cells ($p<0.005$).

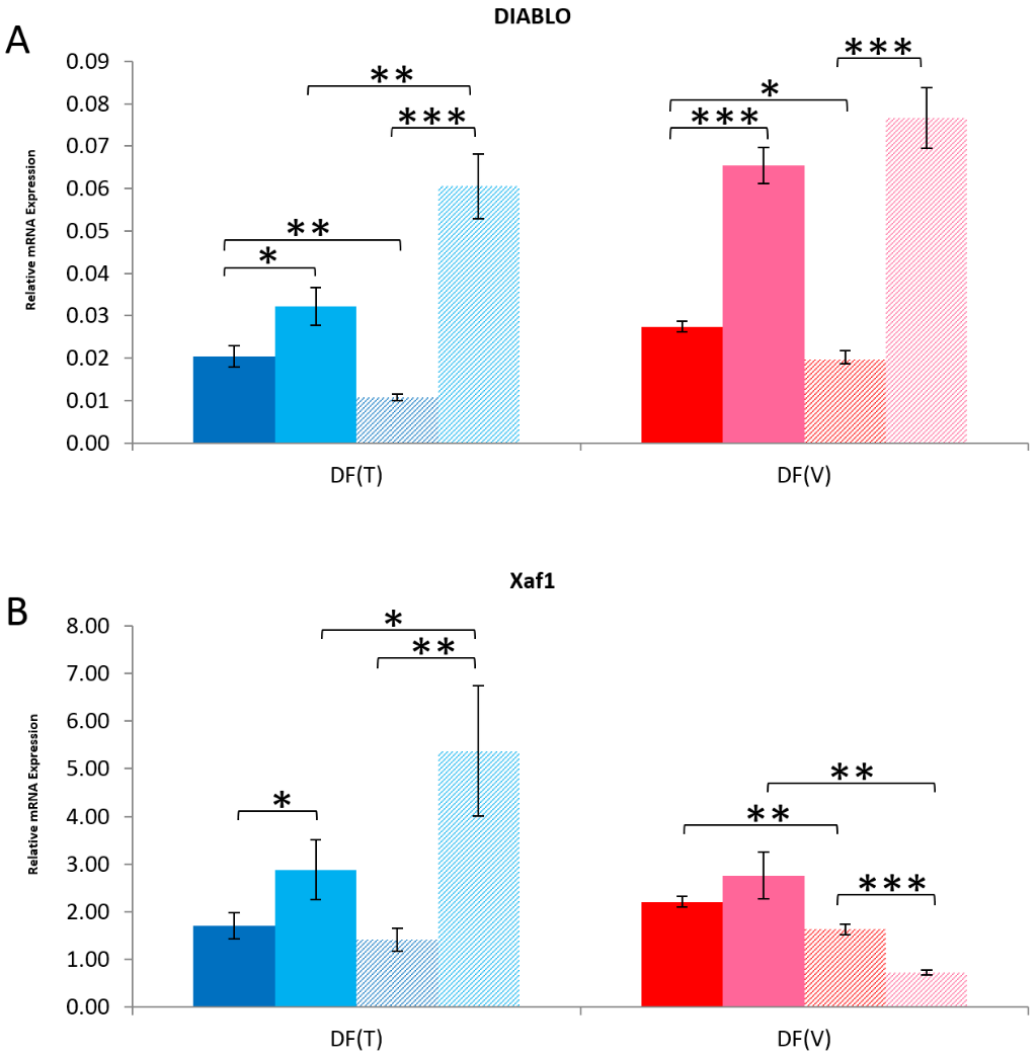


Figure 5.4: Increased expression of IAP antagonists in DF(T) cells with DIABLO increasing and Xaf1 decreasing in DF(V) cells with 17β-oestradiol treatment

*IAP antagonist mRNA transcription expression analysed via qRT-PCR for matching DF(T) and DF(V) cells in unwounded and mechanically wounded conditions, in the presence and absence of 17β-oestradiol normalised against GAPDH. A) mRNA expression for DIABLO. B) mRNA expression for Xaf1. Dark blue – vehicle control DF(T), light blue – 17β-oestradiol treated DF(T), red – vehicle control DF(V), pink – 17β-oestradiol treated DF(V), solid bar – unwounded cells, hashed bar – wounded cells. n=3 donors. Student T-test, * p<0.05, ** p<0.005, *** p<0.0005.*

5.4 Discussion

Limited studies have investigated the role of 17β-oestradiol on IAPs. In MCF7 cells, 17β-oestradiol was shown to protect cells from TNFα induced apoptosis via cIAP1 and cIAP2 (Frasor et al., 2009; Stanculescu et al., 2010). Contradicting information with regards to the effect of 17β-oestradiol on the expression of NAIP has been reported; Yin et al. (2008) demonstrated that NAIP transcript was increased when murine uterine epithelial cells were treated with 17β-oestradiol, while Wall et al. (2013) showed that NAIP transcript decreased. In the rat endometrium 17β-oestradiol has been shown to increase XIAP expression while simultaneously decreasing the expression of DIABLO (Leblanc et al., 2003). This thesis is the first study to investigate the role of XIAP in the viability and caspase 3 activity of DF(T) and matching DF(V) cells in the presence and absence of 17β-oestradiol. It is also the first study investigating the effect of 17β-oestradiol on the expression of mRNA transcripts for IAPs (XIAP, Apollon, NAIP and cIAP2) and their antagonists (DIABLO and Xaf1).

5.4.1 The effect of inhibiting XIAP function on the viability of DF(T) and DF(V) cultures

In the presence of embelin, an XIAP antagonist, the cell viability of matching cultures of DF(T) and DF(V) cells was reduced by approximately 23% and 12% respectively in both unwounded and wounded monolayers (Figure 5.1). Embelin binds to the BIR3 domain of XIAP preventing apoptosis, in a similar way to DIABLO/XIAP binding (Chen et al., 2006; Nikolovska-Coleska et al., 2004). XIAP interacts with caspase 9 via its BIR3 domain, while the region between BIR1 and BIR2 binds to caspase 3 (Deveraux and Reed, 1999; Huang et al., 2001), therefore in the presence of embelin, apoptosis is not inhibited by XIAP. Since the viability of DF(T) and DF(V) cells was reduced when treated with embelin, it would suggest that XIAP is important for cell survival.

Its importance in cell survival appears to be more evident in DF(T) cells compared to matching DF(V) cells, with nearly twice as much cell death in DF(T) cultures compared to DF(V) cultures. Since XIAP protein expression in both DF(T) and DF(V) cells was similar under unwounded and wounding conditions (Figure 3.11 and Figure 3.14), other protective mechanisms may be in place in DF(V) cells compared to matching DF(T) cells.

Stanculescu et al. (2010) demonstrated that in the breast cancer cell line BT-474, cIAP2 is potentially important in cell survival, since cmpd3 (a DIABLO mimetic) reduced cell survival and that mRNA expression for cIAP2 was higher than cIAP1 and XIAP levels. Since cIAP2 mRNA expression was higher in DF(V) cells compared to matching DF(T) cells (Figure 3.7), this could suggest that cIAP2 is more important in the survival of DF(T) and DF(V) cells with a more important role in maintaining cell viability of DF(V) cells compared to matching DF(T) cells.

A study by Ahn et al. (2007) demonstrated that embelin is not only an XIAP antagonist, but can also block NFκB activation in human myeloid KBM5 cells. Using nuclear extracts, NFκB was measured in cells treated in the absence and presence of embelin at different concentrations. With higher concentration, NFκB activity was reduced. This could affect other gene transcription, since IAPs (cIAP1 and cIAP2) are upregulated with NFκB activation, IAP expression may also be modulated by embelin. Therefore, qRT-PRC should be conducted on DF(T) and DF(V) cells to investigate the effect of embelin on IAP expression through possible NFκB activation. A gene micro array would also establish which genes are up-regulated when cells are treated with embelin.

Incubation of embelin treated DF(T) and DF(V) cells in the presence of 17β-oestradiol, improved cell viability, which could suggest that 17β-oestradiol potentially regulates IAP transcription. Therefore, qRT-PCR was carried out to investigate whether 17β-oestradiol regulates IAP levels in DF(T) and DF(V)

cells. To confirm that 17β-oestradiol protection of DF(T) and DF(V) cells is via XIAP, siRNA may be used to block XIAP production.

In unwounded cultures, 17β-oestradiol had a similar protective effect on DF(T) and DF(V) cells (Figure 5.1). However, following mechanical wounding 17β-oestradiol offered a higher degree of protection in DF(T) cells compared with DF(V) cells. Since IAP expression is regulated via NFκB and 17β-oestradiol activates NFκB, it would be interesting to find out whether 17β-oestradiol regulates IAP expression though NFκB activation. A study by Lewis-Wambi and Jordan (2009) demonstrated that 17β-oestradiol could induce or inhibit apoptosis in MCF7 cells. This depended on the cells being deprived of oestradiol. In cells that were long term deprived of oestradiol, treatment with 17β-oestradiol caused a reduction in NFκB, whilst the opposite effect was seen in normal MCF7 cells. Oestrogen receptor expression in DF(T) cells was higher compared to DF(V) cells, therefore 17β-oestradiol may have a different effect on NFκB activity in DF(T) and DF(V) cells. 17β-oestradiol may in DF(T) cells stimulate NFκB activation therefore causing the increase in IAPs, whilst in DF(V) cells, NFκB could be reduced, lowering IAP production. A study by Gionet et al. (2009) demonstrated that expression of ERα in breast cancer cell lines MCF7 and LCC1 caused a reduction in NFκB activation.

Another possible mechanism is that 17β-oestradiol directly inhibits caspase 3 downstream activity. 17β-oestradiol has been shown to reduce caspase 3 activity in human osteoblasts when caspase 3 was induced by TNFα (Bradford et al., 2010). In this paper, the mRNA expression for the IAP Survivin was down regulated when osteoblasts were treated with 17β-oestradiol, which, if caspase 3 activity was reduced, it would have been assumed that IAP levels would increase to prevent apoptosis and caspase 3 activation. However, other apoptosis inducers were down regulated with 17β-oestradiol. This could suggest that in the osteoblast cells, Survivin is not functioning as an IAP. Either other IAPs are regulated by 17β-oestradiol and should be analysed via qRT-PCR, or 17β-oestradiol is modulating caspase 3 activity independently of IAPs. Silencing the IAP genes, or treating cells with DIABLO mimetics would

inhibit IAP function. If caspase 3 activity was reduced by 17β-oestradiol via IAP function, blocking of these IAPs should theoretically prevent caspase 3 reduction. If caspase 3 activity was still reduced, it would indicate that 17β-oestradiol blocks caspase 3 via another pathway.

5.4.2 Effect of Embelin and 17β-oestradiol on caspase 3 activity

In rat brain, it was observed that 17β-oestradiol blocked the cleavage of caspase 3 preventing apoptosis through ERα (Li et al., 2011). Caspase 3 activity was measured by staining cleaved caspase 3. Rats treated with 17β-oestradiol and exposed to trauma showed a decrease in cleaved caspase 3 compared to control. Western blotting demonstrated that ERα expression was increased in traumatised brain, with a further increase in those treated with 17β-oestradiol, whilst no difference was seen in ERβ expression. In chapter 4, qRT-PCR demonstrated that although 17β-oestradiol had no effect on mRNA expression of oestrogen receptors in unwounded DF(T) and DF(V) cells, following mechanical wounding, ERα and ERβ mRNA expression increased in DF(T) cells, while GPR30 and ERα mRNA increased in DF(V) cells in the presence of 17β-oestradiol (Figure 4.5). The level of ERα mRNA was higher in DF(V) cells treated with 17β-oestradiol after mechanical wounding compared to DF(T) cells. Therefore, it is possible that the upregulation of ERα in mechanically wounded DF(T) and DF(V) cells may be a mechanism by which caspase 3 activity is inhibited. Treatment of DF(T) and DF(V) cells with ERα or ERβ agonists would help establish via which receptor 17β-oestradiol may block caspase 3 activity.

In this study, embelin treatment did not increase the basal level of caspase 3 activity of either DF(T) or DF(V) cells under unwounded or mechanically wounded conditions (Figure 5.2). With XIAP inhibition, caspase 3 activity would be expected to increase, as XIAP cannot bind to caspase 3 preventing its activation. However, it has already been established that the other IAPs, cIAP1, cIAP2 and NAIP also interact with caspase 3 preventing its activation (Vucic et al., 2000).

Another possibility is that caspase 3 activity was measured 24 hours after embelin treatment. A study by Xu et al. (2010) demonstrated that human dermal fibroblasts showed increased caspase 3 activity after 12 hours of UVB exposure, decreasing progressively after 48 hours. This shows that caspase 3 activity might have already increased in embelin treated DF(T) and DF(V) cells and returned to basal levels; measuring caspase activity at earlier time points would confirm this. Also, the reduction of cell viability in both DF(T) and DF(V) cells when treated with embelin could have occurred at an earlier time point. Therefore, the protective function of 17β-oestradiol may have been within the first few hours of embelin exposure.

A study by Cheng et al. (2010) demonstrated that embelin alone did not increase caspase 3 activity in non-small lung cancer cell lines. However, embelin enhanced caspase 3 activation when cell were treated with cisplatin, a cancer drug which causes DNA damage leading to cell death (Gonzalez et al., 2001). Levels of XIAP in this cell line was shown to be high, therefore, the concentration of embelin (50µM) used to treat the cells might not have been high enough to affect caspase 3 activity.

Previous studies have shown that endonuclease G (an enzyme that cleaves DNA) and AIF that are localised in the mitochondrion and translocate to the nucleus during apoptotic stress causes chromosomal DNA cleavage independently of caspases (Li et al., 2001; Parrish et al., 2001; Seo et al., 2014). In human embryonic kidney 293 cells, using co-immunoprecipitation, it was shown that cIAP1 but not cIAP2 interacts with the endonuclease G enzyme via its BIR3 domain (Seo et al., 2014). Suggesting that cell viability of DF(T) and DF(V) cells could be decreased due to higher levels of endonuclease G or AIF in the nucleus. Cell fractioning to separate nuclear contents and immunoprecipitation could help establish whether there are higher concentrations of these proteins in embelin treated DF(T) and DF(V) cells.

Recently it has been suggested that embelin is not only an XIAP antagonist, but can also act as an anti-oestrogen (Sumalatha et al., 2014). This was due to the fact that embelin demonstrated a similar function to tamoxifen in decreasing cell migration and proliferation and increasing apoptosis in a breast cancer cell line. Another study by Zhou et al. (2000) demonstrated that in mammary tissue from monkeys, tamoxifen reduced ERα mRNA expression, therefore it would be important to check whether embelin also affects oestrogen receptor expression.

Caspase 3 activity was not affected in unwounded and wounded DF(T) and DF(V) cells when incubated with 17β-oestradiol. This could be due to the time point in which caspase 3 activity was measured, or that 17β-oestradiol only prevents caspase 3 activity when apoptosis is induced (Figure 5.2). mechanically wounding cells did not affect cell viability (Figure 5.1), nor was there any significant difference in caspase 3 activity between wounded and unwounded DF(T) and DF(V) cells.

Interestingly, when DF(T) cells were co-incubated with 17β-oestradiol and embelin together, the amount of caspase 3 activity decreased in both unwounded and mechanically wounded cells, while no significant changes were seen in either unwounded or mechanically wounded DF(V) cells (Figure 5.2). This may suggest that 17β-oestradiol may oppose the effect of embelin on caspase 3 in DF(T) cells. A study on non-small lung cancer cell lines has shown that a 2 hour pre-treatment of embelin is enough to reduce cell numbers after 24 hours (Cheng et al., 2010). Therefore, the effect of embelin may be more rapid in DF(V) cells, and caspase 3 activity affected at earlier time points. After 24 hours, caspase 3 activity might return to normal levels in DF(V), therefore any effect of 17β-oestradiol would not be seen. Since 17β-oestradiol alone did not reduce caspase 3 activity in unwounded and mechanically wounded DF(T) and DF(V) cells, protective effects of 17β-oestradiol may only be evident when cells are stressed (e.g. via TNFα or UV), suggesting why caspase 3 activity was not affected with 17β-oestradiol alone.

Although the pathway by which 17β-oestradiol is acting is unknown, oestrogen receptor expression in DF(T) and DF(V) was altered in the presence of 17β-oestradiol following mechanical wounding (Figure 4.5). Since XIAP was important for cell survival (Figure 5.1), blocking XIAP could increase cell stress, which could potentially affect oestrogen receptor expression. To determine which oestrogen receptor is involved protecting against a reduction in cell viability and/or reducing caspase 3 activity in DF(T) cells, the use of specific oestrogen receptor agonists or antagonists or silencing the oestrogen receptor genes using siRNA would establish whether 17β-oestradiol protects cells independently of oestrogen receptors, or via oestrogen receptors, and if so, which one(s).

5.4.3 The effect of 17β-oestradiol on IAP mRNA expression

To determine whether 17β-oestradiol has a direct effect on IAP expression *in vitro*, the expression of mRNA for XIAP, Apollon, cIAP2 and NAIP was analysed in unwounded and mechanically wounded cell cultures in both the presence and absence of 17β-oestradiol.

In DF(T) cells, XIAP mRNA expression in unwounded and mechanically wounded cells increased in the presence of 17β-oestradiol, and while 17β-oestradiol increased XIAP mRNA in unwounded DF(V) cells, in mechanically wounded DF(V) cells, 17β-oestradiol decreased XIAP expression (Figure 5.3). This could be the reason why 17β-oestradiol co-treatment with embelin on caspase 3 activity was selective to DF(T) cells. Since 17β-oestradiol stimulated an increase in XIAP mRNA expression, a subsequent increase in XIAP protein may counteract the effect of embelin and decrease caspase 3 activity (Figure 5.2). This trend was seen in DF(T) and DF(V) cells, where both unwounded and wounded DF(T) caspase 3 activity was reduced, and only in unwounded DF(V) cells caspase 3 activity was reduced.

No studies have been conducted to investigate the effect of 17β-oestradiol on Apollon expression. Opposite effects were seen in DF(T) and DF(V) cells incubated with 17β-oestradiol; mRNA Apollon transcript were increased in

unwounded and mechanically wounded DF(T) cells, but decreased in corresponding DF(V) cells (Figure 5.3). This would suggest that Apollon is not involved in protecting DF(V) cells, but could be involved in protecting DF(T) cells, since mechanically wounding and 17β-oestradiol both decease Apollon expression in DF(V) cells. However, Apollon could be important in regulating cell number. With a decrease in Apollon, more cells are likely to undergo apoptosis, however, since 17β-oestradiol did not modulate cell proliferation of either DF(T) or DF(V) cells (Figure 4.2), this suggests that Apollon is not involved in proliferation.

17β-oestradiol had no effect on cIAP2 mRNA expression in unwounded DF(T) cells, but increased cIAP2 expression in wounded DF(T) cells. While in DF(V) cells, 17β-oestradiol decreased cIAP2 mRNA expression in unwounded cells, and had no effect in wounded DF(V) cells (Figure 5.3). Expression of cIAP2 was higher in DF(V) cells compared to matching DF(T) cells. This suggests that cIAP2 has a more dominant function in DF(V) cells, compared to DF(T). 17β-oestradiol has been shown to protect MCF7 cells from TNFα induced apoptosis via cIAP2 (Frasor et al., 2009; Stanculescu et al., 2010). Since mRNA expression of cIAP2 was higher in DF(V) cells, it could suggest the reason why the cell viability of DF(V) cells was not reduced as much as corresponding DF(T) cells when incubated with embelin.

The expression of NAIP mRNA expression was increased in unwounded and mechanically wounded DF(T) cells in the presence of 17β-oestradiol, while expression remained unchanged in corresponding unwounded and mechanically wounded DF(V) cells (Figure 5.3). A study by Yin et al. (2008) demonstrated by qRT-PCR that in the uterine epithelial cells of ovariectomised mice, 17β-oestradiol induced the expression of NAIP transcript, and that active caspase 3 and caspase 9 were abolished in the presence of 17β-oestradiol *in vivo*. This supports the data obtained with DF(T) cells. The opposite effect of 17β-oestradiol seen in DF(V) cells is supported by a study by Wall et al. (2013), demonstrating that 17β-oestradiol treatment *in vivo*, caused an increase in apoptosis in ovariectomised mouse uterine

epithelial cells, detected by a TUNEL assay, and that NAIP mRNA transcript were decreased as quantitated by qRT-PCR. The increase in NAIP mRNA transcript in DF(T) but not DF(V) cells in the presence of 17β-oestradiol could be attributed to the difference in oestrogen receptor expression. Expression of GPR30 was higher in DF(T) cells compared to matching DF(V) cells in both unwounded and mechanically wounded cells, in the presence and absence of 17β-oestradiol (Figure 4.4). However, to confirm the increase of NAIP mRNA transcript in DF(T) cells was due to GPR30, the gene can be silenced, and the effect of 17β-oestradiol on NAIP mRNA expression can be assessed.

DIABLO is an IAP antagonist (Cossu et al., 2009; Verhagen et al., 2001; Verhagen et al., 2000), and is important in regulating IAP levels. In DF(T) and matching DF(V) cells, expression of DIABLO mRNA increased in the presence of 17β-oestradiol (Figure 5.4). The increase in DIABLO mRNA expression when DF(T) and DF(V) cells were incubated with 17β-oestradiol, could be due to the up regulation in IAP expression in DF(T) cells, therefore the increase would be to maintain IAPs at a specific level, while in DF(V) cells, DIABLO could be the cause for the reduction in IAP expression, since IAP expression in higher in DF(V) cells compared to matching DF(T) cells. After 24 hours following mechanical wounding, no change in the protein expression of DIABLO was seen (Figure 3.15) however, higher expression was found in DF(V) cells compared to DF(T) (Figure 3.16). Mechanical wounding caused a decrease in mRNA expression in both DF(T) and DF(V) cells, which could suggest that apoptosis needs to occur to get rid of any damaged cells, but also to regulate the amount of protein. After immediate wounding, DIABLO protein expression significantly increased, for DIABLO to return to unwounded levels, mRNA expression might need to decrease. Increased expression of DIABLO mRNA with 17β-oestradiol could also indicate that DIABLO has other functions when activated by 17β-oestradiol. The fact that in unwounded and mechanically wounded DF(V) cells, DIABLO expression increased but the majority of IAP levels either remained the same or decreased, would have suggested that in DF(V) cells 17β-oestradiol may induce apoptosis. However,

initial expression of IAP transcript was high in DF(V) cells, and cell viability was not affected with 17β-oestradiol alone.

A study by Yao et al. (2007) looked at the effect of 17β-oestradiol on the IAP antagonist DIABLO protein in neuronal cell cultures, and showed that when neuronal cells were stressed by β-Amyloid which increased DIBALO expression, 17β-oestradiol brought DIABLO protein levels back to normal levels found in the mitochondria and cytoplasm. When cells are undergoing apoptosis, expression of DIABLO is increased in the cytosol and decreased in the mitochondria (Yao et al., 2007). Since the location of DIABLO is important for its function, it would be important to analyse where in the cell DIABLO is expressed, whether it is in the nucleus and active or in the cytoplasm. To demonstrate this, a mitochondria/cytosol fractioning kit may be used to separate proteins that are found in the mitochondria and cytoplasm. DF(T) and DF(V) cells would be incubated in the presence or absence of 17β-oestradiol, then fractioned. Western blotting of the two separate regions would establish the localisation of DIABLO. Also using double immunoprecipitate, it would be possible to confirm whether DIABLO is bound to any IAPs. A study by Leblanc et al. (2003) also demonstrated that in rat uterine endometrial cells, 17β-oestradiol regulated the expression levels for DIABLO which regulated XIAP expression. However, in their study, incubation with 17β-oestradiol caused DIABLO expression to decrease. This indicates that different species and different cells express and regulate IAPs differently. To ensure that DIABLO is binding to IAPs and functioning as an IAP antagonist, co-immunoprecipitation can be carried out on DIABLO and stained against IAPs and vice versa to make sure that DIABLO has bound to IAPs preventing their function.

Xaf1 is a direct XIAP antagonist (Liston et al., 2001), there have been no previous studies that have looked at the effect of 17β-oestradiol on the expression of Xaf1. Changes in mRNA expression of Xaf1 mirrored the expression of XIAP when cells were incubated with 17β-oestradiol in both DF(T) and DF(V) cells (Figure 5.3 and Figure 5.4). Since Xaf1 is a direct

inhibitor of XIAP, and XIAP inhibits apoptosis. However, it could suggest that in DF(T) and DF(V) cells Xaf1 mRNA expression is required to ensure that XIAP levels are maintained. A study by Xia et al. (2006) using mice fibroblasts derived from a XIAP knockout animal, showed that Xaf1 still sensitised these cells to TNFα, suggesting there is another mechanism for Xaf1 to promote apoptosis independently of XIAP. This could suggest that in DF(T) and DF(V) cells XIAP and Xaf1 have different roles that are mediated by 17β-oestradiol, this could be through ERβ, since ERβ is up regulated in DF(T) and down regulated in DF(V) cells when mechanically wounded and in the presence of 17β-oestradiol.

Effect of 17β-oestradiol in unwounded cells on:	DF(V) cells compared to DF(T) cells
NAIP	Higher
cIAP2	Higher
Apollon	Lower
XIAP	Same
DIABLO	Higher
Xaf1	Same

Effect of 17β-oestradiol in wounded cells on:	DF(V) cells compared to DF(T) cells
NAIP	Same
cIAP2	Higher
Apollon	Lower
XIAP	Lower
DIABLO	Same
Xaf1	Lower

Table 5.2: Expression levels of IAP and IAP antagonist mRNA in DF(V) cell compared to DF(T) when treated with 17β-oestradiol

A comparison between DF(V) and DF(T) cells in mRNA expression of the IAPs NAIP, cIAP2, Apollon and XIAP, their antagonists DIABLO and Xaf1 in DF(V) cells in comparison to DF(T) cells in unwounded and mechanically wounded cells in the presence of 17β-oestradiol.

5.5 Conclusion

In this chapter, it was demonstrated that embelin an XIAP antagonist reduces cell viability in both unwounded and wounded DF(T) and DF(V) cells derived from the same donor. Furthermore, embelin was more effective at reducing cell viability in DF(T) cells compared to DF(V) cells. This would suggest that XIAP function is important in maintaining cell viability particularly in DF(T) cultures. 17β-oestradiol increased cell viability when incubated with unwounded and mechanically wounded DF(T) cells treated with embelin, while it only increased viability in corresponding unwounded DF(V) cells.

Interestingly, caspase 3 activity was not increased in either unwounded or mechanically wounded DF(T) and DF(V) cells treated with embelin. 17β-oestradiol reduced caspase 3 activity in DF(T) cells incubated with embelin, while this effect was not seen in DF(V) cells.

Incubation with 17β-oestradiol up regulated mRNA expression of XIAP, Apollon and NAIP in both unwounded and mechanically wounded DF(T) cells, but only the up regulation of cIAP2 in mechanically wounded cells. However, in mechanically wounded DF(V) cells, 17β-oestradiol reduced the mRNA expression of XIAP and Apollon; but unwounded DF(V) cells, it increased the expression of XIAP and decreased the expression of cIAP2. 17β-oestradiol had no effect on NAIP expression in both unwounded and mechanically wounded DF(V) cells. Since 17β-oestradiol had different effects on DF(T) and DF(V) cells in terms of IAP expression, it would suggest that 17β-oestradiol protects DF(T) cells through the up regulation of IAPs. However, since IAPs were mainly reduced in DF(V) cells, with the exception of XIAP, 17β-oestradiol may protect cells through XIAP expression alone.

Incubation with 17β-oestradiol up regulated mRNA expression of DIABLO in unwounded and mechanically wounded DF(T) and DF(V) cells. It also increased Xaf1 expression in unwounded and mechanically wounded DF(T) cells, while in DF(V) cells, 17β-oestradiol had no effect on Xaf1 mRNA expression in unwounded cells, but decreased expression in mechanically wounded cells. Since Xaf1 binds to XIAP to prevent XIAP function, it could be assumed that in DF(V) cells, XIAP is an important IAP, since the decrease in Xaf1 mRNA transcript could mean that there are less Xaf1 to bind to XIAP.

Therefore, IAPs transcript appear to be differentially regulated by 17β-oestradiol in both unwounded and mechanically wounded DF(T) and DF(V) cells, which may explain their differences in terms of caspase 3 activity and protection against a reduction in cell viability. Taken together, this data suggests that 17β-oestradiol may have a role in protecting dermal fibroblasts against apoptosis when IAP function is compromised.

Chapter 6

DISCUSSION AND CONCLUSION

6 Discussion and Conclusion

6.1 Summary of Discussion

IAPs are proteins that inhibit apoptosis, there are eight mammalian IAPs that have been identified (Wei et al., 2008). IAPs function by binding to caspases, preventing them from being cleaved and subsequently activating the caspase cascade, which eventually leads to apoptosis (Deveraux and Reed, 1999; Deveraux et al., 1998). DIABLO is a general IAP antagonist (Verhagen et al., 2000) that binds either directly to IAPs or interferes with caspase 9 binding, while Xaf1 is a direct XIAP antagonist (Liston et al., 2001). Subsequently, there have been other IAP antagonists identified, Sept4/ARTS like Xaf1, targets XIAP (Gottfried et al., 2004; Larisch et al., 2000) while Omi/HtrA2 acts in a similar way to DIABLO and cleaves various IAPs, including cIAP1, cIAP2 and XIAP (Verhagen et al., 2002; Yang et al., 2003). Expression of human IAPs appears to be cell and tissue specific (Vischioni et al., 2006). The control of apoptosis is essential in wound healing and tissue regeneration, but the role of IAPs, particularly in human skin has not been investigated. An improved rate and quality of wound healing in haired human skin has long been acknowledged (Jahoda and Reynolds, 2001). Although this has been attributed to the hair follicle stem cells, “non-haired” human skin also contains hair follicles, although these produce small, fine and unpigmented vellus hairs. In studies of human skin, there is much variation between donors. Therefore, this study has used matching skin biopsies of human skin containing terminal or vellus hair follicles from the same donor.

Oestrogens are also important in regulating wound healing in the skin (Ashcroft et al., 1997a; Ashcroft et al., 1999). In the skin and hair follicles, oestrogen receptors have been identified in both humans and mouse (Campbell et al., 2010; Kwon et al., 2004; Markiewicz et al., 2013; Merlo et al., 2009; Moverare et al., 2002; Ohnemus et al., 2005; Thornton et al., 2003a, b). A more predominant expression of ERβ in human skin and hair follicles would suggest that in wound healing, 17β-oestradiol improves wound healing via

ERβ (Campbell et al., 2010). The re-establishment of 17β-oestradiol in post menopausal female donors via topical application improved wound healing (Ashcroft et al., 1999). It has been established that the anagen phase of the hair cycle promotes wound re-epithelialisation in humans (Ansell et al., 2011); and that 17β-oestradiol regulates the hair follicle cycle (Chanda et al., 2000), therefore 17β-oestradiol could improve wound healing in hairy skin via its role in the hair follicle cycle, which in turn, regulates wound healing.

The hair cycle is an excellent model to study regeneration and tissue remodelling, due to the lower region of the hair follicle undergoing cycles of apoptosis, regeneration and remodelling recapitulating the events of late embryogenesis (Commo and Bernard, 1997; Hashimoto et al., 2001; Tobin et al., 2003). In a murine model, it is easier to study the individual hair follicle cycle stages since anagen can easily be induced compared to humans.

In this study, it has been established that the expression of IAPs (XIAP, Apollon, cIAP1 and cIAP2) differed in various compartments of the skin and hair follicle at distinct stages of the hair cycle (Figure 2.4 and Table 2.4). Interestingly, the expression of these IAPs was found in the dermis at day 5 after depilation (early to mid anagen). The expression could be due to the effect of depilation on the skin and hair follicle, since depilation causes a wound healing response in the skin and hair follicle (Matsuo et al., 2003). However, since it has already been established that wound healing is improved in skin containing terminal hair follicles (Ansell et al., 2011), the improved wound healing response could be due to the expression of these IAPs during this stage of the hair cycle. These IAPs were also found in stem cell niches in the skin and hair follicle, which would indicate their importance in protecting cells that are required for regeneration especially during wound healing (Ito et al., 2005; Levy et al., 2007; Plikus et al., 2012). Therefore, these IAPs might not only be important in regulating the hair follicle cycle, but are also important in contributing to a wound healing response. It was important to establish whether these IAPs were also expressed in human skin and potentially regulate a wound healing response. One study by Fuchs et al.

(2013) demonstrated the importance of XIAP in murine wounds. The inhibition of XIAP via gene knockout delayed wound re-epithelialisation, which suggested the importance of IAPs in particular XIAP in wound healing.

To date, this is the first study to compare the expression of IAPs and their antagonists in human skin derived from terminal (scalp) and vellus (facial) hair bearing skin of the same individual. Since studies have demonstrated the importance of terminal hair follicles in wound healing (Ansell et al., 2011; Ito and Cotsarelis, 2008; Jahoda and Reynolds, 2001), and that wounds heal better in hairy region compared to “non hairy” regions (Jahoda and Reynolds, 2001; Martinot et al., 1994), it was important to establish whether this was due to difference in IAP expression.

The majority of hair follicles in the scalp are in the anagen phase of the hair cycle, 90% of hair follicles are terminal hair follicles while the rest are vellus hair follicles (Whiting, 1993, 1996). Expression of IAPs was similar in the dermis between terminal and vellus hair bearing skin, with the exception of cIAP2 which had a lower expression in vellus hair bearing skin compared to matching terminal hair bearing skin, however, expression in the epidermis was higher in terminal hair bearing skin compared to matching vellus hair bearing skin for cIAP2, NAIP and XIAP. This could potentially be due to epidermal thickness, since the epidermis is thicker in terminal hair bearing skin compared to corresponding vellus hair bearing skin (Figure 2.7). A thicker epidermis would suggest that there are more keratinocytes, therefore regulation of cell death needs to be tightly controlled. This could also be the reason why terminal hair bearing skin heals better with less scarring compared to non-hairy skin.

The main difference in IAP expression between human skin and murine skin was seen in the dermis, where expression was lower in murine dermis, however, it was difficult to compare between mouse and human skin since it was difficult to establish which stage of the hair cycle the terminal hair follicles were in when stained with IAP antibodies. In the dermis, the main cells are the fibroblasts; in human skin, there are a higher number of fibroblasts in the

dermis compared to murine dermis (Dams et al., 2011). This could explain why there was a higher expression of IAPs in human dermis compared to mouse dermis. Also, if the hair follicle contributes to the expression of IAPs in the skin, hair follicles in mice grow in trios (Adelson et al., 1997), with smaller hair follicles in mice compared to humans (Griffin et al., 2006).

Interestingly, the expression of DIABLO and Xaf1 in both human (Figure 2.9 and Table 2.8) and murine skin (Figure 2.5 and Table 2.5) showed high expression. DIABLO (Cossu et al., 2009; Verhagen et al., 2001; Verhagen et al., 2000) and Xaf1 (Liston et al., 2001) are IAP antagonists, therefore promote apoptosis. Therefore expression in the skin and hair follicle should theoretically be low, however, DIABLO was expressed in abundance, which would suggest that DIABLO might have another role aside from inhibiting IAP function. A study by Kim et al. (2006) demonstrated that DIABLO interacting with Survivin in the nucleus at the G2/M arrest caused apoptosis. This study was conducted in prostate cancer cells, DIABLO may potentially interact in normal cells in a different manner. Since the interaction was during a specific cell cycle stage, DIABLO may interact with IAPs to promote cell cycling.

Since fibroblasts are important in wound healing and differences were seen in the dermis between terminal and vellus hair bearing skin, dermal fibroblasts derived from terminal and vellus hair bearing skin were cultured. There have been different studies that have demonstrated that dermal fibroblasts derived from different regions have different properties, and that dermal fibroblasts from the same region, but derived from either the reticular or papillary layer were also different (Chipev and Simon, 2002; Schafer et al., 1985; Sorrell et al., 2004; Sorrell and Caplan, 2004). However, no studies have compared different properties related to wound healing between dermal fibroblasts from similar regions either containing terminal (scalp – DF(T)) or vellus (facial – DF(V)) hair follicles from the same donor. These differences could contribute to why there is a difference in wound healing between hairy and non-hairy regions. Since DF(T) and DF(V) cells have a different morphology, a hypothesis from the study conducted by Sorrell and Caplan (2004) suggests

that granular and more complex cells could relate to a higher production of TGFβ1. TGFβ1stimulates the production of more collagen and increases cell proliferation (Jutley et al., 1993; O'Leary et al., 2004), which may account for the differences seen in wound healing responses between hairy and non hairy regions. This could easily be shown by comparing TGFβ1 secretion using ELISA between DF(T) and DF(V) cells, also mRNA expression could also be analysed for TGFβ1.

Therefore, from the results seen in this thesis, DF(V) cells are more granular than corresponding DF(T) cells (Figure 3.4), suggesting that this could contribute to the difference in wound healing responses. Vellus hairs are fine and unpigmented (Vogt et al., 2007), therefore difficult to see and remove when culturing dermal fibroblasts. This therefore could potentially lead to dermal papilla and dermal sheath cells being cultured among dermal fibroblasts from the papillary layer which could also contribute to the difference in morphology. This would not be apparent from terminal hair bearing skin, since hair follicles are removed before culture. Specific markers that differentiate between fibroblasts derived from different regions could be used to assess the purity of the cultures. Morphology should have been analysed not only between DF(T) and DF(V) cells, but also between mechanically wounded and unwounded cells to establish if cell morphology changes in response to wounding. Whilst there was no difference between DF(T) and DF(V) cells in terms of cell migration, DF(V) cells proliferate at a higher rate (Figure 3.5 and Figure 3.6 respectively). The difference in morphology which could attribute to the difference in proliferation rate could be influenced by the hair follicle. The hair follicle dermal sheath could send out signalling molecules which has caused hair follicle fibroblasts to adapt. With the presence of hair follicles, there are more fibroblasts that could provide different functions such as secretion of TGFβ1, and therefore the fibroblasts in the dermis from vellus hair bearing skin potentially need to compensate from the smaller and less available vellus hair follicle. Therefore, it would interesting to culture non contaminated dermal sheath cells from vellus and terminal hair follicles, and

analyse if there are any morphological differences. This hypothesis, would suggest that the hair follicle influences the dermal fibroblast population, however, it could be possible that the dermal fibroblast influence the type of hair follicles growing in the skin. A gene micro array would establish which genes are upregulated in DF(T) and DF(V) cells, and whether there is a difference which could influence hair follicle production.

In addition to migration and proliferation, both important aspects of wound healing (Jaffe et al., 2012), the regulation of apoptosis is also critical in wound healing. This is the first study to compare expression of IAPs and their antagonists in human dermal fibroblasts cultured from terminal and vellus hair bearing skin of the same donor. IAP mRNA expression levels in DF(V) cells were higher compared to DF(T) cells with the exception of XIAP and its direct antagonist Xaf1, which showed similar expression between the two cell types (Figure 3.7). When these cells were mechanically wounded, expression levels decreased, with the exception of XIAP in DF(V) cells, which increased (Figure 3.8), causing mRNA levels of XIAP to be higher in DF(V) cells compared to matching DF(T) cells. From the study by Fuchs et al. (2013), it was demonstrated that XIAP is important in wound healing, in particular wound closure. The increase in XIAP levels in DF(V) cells but not DF(T) cells could indicate the importance of XIAPs in wound healing. Even though expression of XIAP in DF(T) cells did not increase, terminal hair follicles are found in the skin from which these fibroblasts are derived from, therefore the dermal sheath fibroblasts could contribute to wound healing, therefore, the dermal fibroblasts response to mechanical wounding could be influenced by their anatomical region. It would be beneficial to examine the expression levels of IAPs in dermal sheath cells extracted from terminal and vellus hair follicles, and how mechanical wounding may alter IAP expression. Extracting vellus hair follicles for dermal sheath cell culture is difficult to their size and pigmentation, however intermediate hair follicles are more likely to be isolated an analysed (Miranda et al., 2010).

DF(T) and DF(V) cells were incubated with 17β-oestradiol to compare responses in terms of migration and proliferation. In this study it was demonstrated that 17β-oestradiol had no effect on dermal fibroblast proliferation but accelerated migration for DF(T) cells only (Figure 4.3). Migration is important in wound healing to allow fibroblasts to spread across the wound site to produce collagen and rebuild the ECM (Bello and Phillips, 2000; Park et al., 2011). Since wounds heal better in terminal hair bearing skin, the effect of 17β-oestradiol on migration could suggest that DF(T) cells are able to migrate to wounded sites quicker, and since potentially TGFβ1 secretion is regulated wounds heal better with less scarring. However, to understand responses of 17β-oestradiol in DF(T) and DF(V) cells under wounded and non wounded conditions, oestrogen receptor expression was analysed.

With mechanical wounding, expression of the oestrogen receptors (ERα, ERβ and GPR30) decreased in DF(T) cells, while only ERβ decreased in DF(V) cells; so while mRNA expression of ERα and GPR30 was higher in DF(T) cells, after wounding, ERα mRNA expression was higher in DF(V) cells (Figure 4.4). In the presence of 17β-oestradiol, oestrogen receptor expression did not change in unwounded dermal fibroblasts, however, following mechanical wounding ERα and ERβ mRNA increased in DF(T) cells while in DF(V) cells ERα and GPR30 mRNA increased causing GPR30 expression to be similar between DF(T) and DF(V) cells with higher expression of ERα and lower levels of ERβ in DF(V) cells compared to DF(T) cells (Figure 4.5). Changes in oestrogen receptor expression would suggest that fibroblast responses to 17β-oestradiol needs to be tightly regulated. Since migration in the presence of 17β-oestradiol was accelerated only in DF(T) cells, this could be related to expression of oestrogen receptors. In mechanically wounded conditions, in the presence of 17β-oestradiol, ERβ mRNA expression increased to a higher levels than those seen in DF(V) cells, which could suggest that accelerated migration is through ERβ. Previous studies have shown that accelerated migration in dermal fibroblast was through ERα, however these studies where

conducted in fibroblasts cultured from female abdominal skin (Stevenson et al., 2005; Stevenson et al., 2009). However, to solidify this claim, it would be important to assess migration of DF(T) and DF(V) cells in the presence of oestrogen receptor agonists or antagonists, or another method by which the gene for the different oestrogen receptors are silenced using siRNA.

As previously mentioned, IAPs are important in wound healing, and a study by Leblanc et al. (2003) demonstrated that IAPs and their antagonists in particular XIAP and DIABLO respectively are regulated by 17β-oestradiol. Therefore, in this current study, it was demonstrated that 17β-oestradiol did regulate IAP levels in both unwounded and mechanically wounded dermal fibroblasts; in DF(T) cells, 17β-oestradiol increased mRNA expression of the majority of IAPs while in DF(V) cells, IAP expression was mainly reduced (Figure 5.3). IAP expression is regulated by NFκB therefore, changes or differences in NFκB activation between DF(T) and DF(V) cells need to be studied since the effect of 17β-oestradiol on IAP levels could potentially be through NFκB.

Expression of IAPs was higher in unwounded and mechanically wounded DF(V) cells, however, in the presence of 17β-oestradiol, Apollon and XIAP expression was altered to be lower. This could be due to the importance of the dermal fibroblasts in maintaining the hair follicle, in terminal hair bearing skin, larger numbers of larger hair follicles need to be maintained, and therefore with higher IAP expression, cells important for supporting the growth of the hair follicle are regulated. In vellus hair bearing skin, expression of Apollon and XIAP was lower suggesting that these cells are more likely to undergo apoptosis. With a higher proliferation rate and slower migration in DF(V) cells compared to DF(T) cells, fibroblasts may congregate in a small area instead of spreading out in the wound therefore, fibroblasts need to undergo apoptosis to prevent accumulation and excess production of collagen.

Cell viability of both DF(T) and DF(V) cells was reduced in the presence of embelin an XIAP inhibitor (Nikolovska-Coleska et al., 2004) (Figure 5.1), however, the reduction in cell viability was greater in DF(T) compared to

matching DF(V) cells. This suggests that XIAP is important in maintaining cell viability, since cell viability of DF(V) cells was not reduced to the same levels seen in DF(T) cells, it would suggest that there are other mechanisms that are in place to protect DF(V) cells that might not be present in DF(T) cells, e.g. the expression of other IAPs protecting the cells. The addition of 17β-oestradiol to embelin treated cells improved cell viability in unwounded and mechanically wounded DF(T) cells, while only in unwounded DF(V) cells. This correlates with mRNA expression of XIAP, in unwounded and mechanically wounded DF(T) cells, XIAP mRNA increased with 17β-oestradiol treatment (Figure 5.3), however, in DF(V) cells, 17β-oestradiol increased expression in unwounded cells but decreased expression in wounded DF(V) cells (Figure 5.3); providing evidence that cell protection by 17β-oestradiol is via an increase in XIAP expression.

The ability of IAPs to prevent apoptosis is by interacting with caspases preventing their activation. Caspase 3 is the effector caspase which when activated leads to cell death (Strasser et al., 2000). Caspase 3 activity was measured in DF(T) and DF(V) cells in the presence of embelin and 17β-oestradiol (Figure 5.2). No significant differences were seen in caspase 3 activity in dermal fibroblasts treated with embelin, however, with embelin and 17β-oestradiol treatment, caspase 3 activity was reduced in both unwounded and mechanically wounded DF(T) cells. This could also be related to XIAP mRNA expression, since higher levels of XIAP might protect against caspase 3 activation and in DF(T) cells in the presence of 17β-oestradiol, XIAP mRNA expression was higher compared to matching DF(V) cells. A similar trend was seen in unwounded DF(V) cells, also correlating to XIAP mRNA expression. It is difficult to assess via which receptor 17β-oestradiol protects dermal fibroblasts. The trend in ERβ expression between mechanically wounded and unwounded DF(T) and DF(V) cells, could suggest that 17β-oestradiol acts through ERβ. However, to solidify this claim, further experiments need to be carried out using either oestrogen receptor agonists, or blocking oestrogen receptor expression by antagonists, or silencing the oestrogen receptor genes

using siRNA and seeing the effect it has on caspase 3 activity in DF(T) and DF(V) cells.

There are other IAPs expressed in DF(T) and DF(V) cells which may contribute to the differences seen between DF(T) and DF(V) cells. Apollon mRNA expression in DF(T) and DF(V) cells was relatively high (Figure 3.7). This could suggest that this protein is important in dermal fibroblasts. Expression of Apollon mRNA was higher in DF(V) cells compared to matching DF(T) cells. Protein expression increased in DF(T) cells immediately following mechanical wounding returning to unwounded levels after 24 hours, while it remained constant in DF(V) cells (Figure 3.10). A higher constant expression of Apollon between unwounded and mechanically wounded cells could potentially suggest that in DF(V) cells Apollon is more important in protecting against cell death, since basal levels of Apollon were higher in DF(V) cells (Figure 3.11). So whilst Apollon may potentially protect DF(V) cells against embelin, expression of Apollon mRNA decreased in the presence of 17β-oestradiol, suggesting that 17β-oestradiol did not protect DF(V) cells via Apollon.

NAIP mRNA expression was the lowest of the IAPs in DF(T) and DF(V) cells (Figure 3.7), which would suggest that it might not be important in dermal fibroblasts. Its localisation is specific to just outside the nucleus, its increase in expression by 17β-oestradiol suggests that it is important in cell survival in DF(T) but not necessarily DF(V) cells since 17β-oestradiol only increased NAIP mRNA expression in DF(T) cells not DF(V) cells (Figure 3.8). NAIP requires ATP to bind to caspase 9 (Davoodi et al., 2010b), which causes a conformational change (Davoodi et al., 2004). Expression of NAIP could have decreased following mechanical wounding in DF(V) cells and remained low due to NAIP being used up and not replenished. Cell viability should be assessed with NAIP gene silencing to see if 17β-oestradiol protects cell through NAIP in DF(T) cells, reducing caspase 3 activity since NAIP mRNA transcript increased in DF(T) cells in the presence of 17β-oestradiol.

6.2 Summary of Conclusion

In conclusion, this study has demonstrated that IAPs and their antagonists are present in both mouse and human skin by immunofluorescent staining. Expression of IAPs in the mouse model varied in the skin and hair follicle at different stages of the hair cycle. In human skin, since it was difficult to study the expression of IAPs at different stages of the hair cycle, a difference between vellus and terminal hair bearing skin was analysed. The difference observed between terminal and vellus hair bearing skin would suggest that IAPs are regulated differently, which could contribute to the type of follicle maintained and/or the difference in the rate and quality of wound healing. Since ethically it would be difficult to induce anagen and study the hair cycle in humans, dermal fibroblasts from terminal and vellus hair bearing skin from similar anatomical regions from the same donor were cultured and studied.

From the different experiments comparing DF(T) and DF(V) cells, it was concluded that DF(T) and DF(V) cells not only showed different characteristics in terms of size and cell complexity, but also in their proliferation rate. However, no differences were observed between DF(T) and DF(V) in cell migration. Their responses to mechanical wounding in culture and to incubation with 17β-oestradiol was also different. Whilst both DF(T) and DF(V) cells expressed the IAPs NAIP, cIAP2, Apollon and XIAP, and the antagonists DIABLO and Xaf1, expression of these mRNA transcripts was higher in DF(V) cells compared to DF(T) cells with the exception of XIAP and its direct antagonist Xaf1. The protein expression of IAPs was similar between unwounded DF(T) and DF(V) cells with the exception of Apollon, which was higher in DF(V) cells. This could suggest that Apollon has an important role in DF(V) cells compared to matching DF(T) cells.

Protein expression of ERα and ERβ in the skin and hair follicle was different between terminal and vellus hair bearing skin. 17β-oestradiol had no effect on DF(T) and DF(V) proliferation but accelerated migration in DF(T) cells only. Higher mRNA expression of GPR30 and ERα was seen in DF(T) cells compared to matching DF(V) cells, whilst expression of ERβ was similar. This

could suggest that DF(T) cells react quicker (via GPR30) to 17β-oestradiol compared to DF(V) cells. Incubation with 17β-oestradiol had no effect on oestrogen receptor transcript levels in unwounded DF(T) and DF(V) cells, but did modulate oestrogen receptor expression in mechanically wounded cells, suggesting that in a wound healing response 17β-oestradiol controls different aspects of wound healing in DF(T) and DF(V) cells, such as modulating collagen synthesis, and adhesion of cells.

Embelin, a potent XIAP antagonist reduced cell viability in both DF(T) and DF(V) cells, with more cell death seen in DF(T) cells compared to matching DF(V) cells. This suggests that XIAP is important in maintaining cell viability; however, its significance is more prominent in DF(T) cells than DF(V) cells, or DF(V) cells have other mechanisms in place to protect cells. 17β-oestradiol protected both DF(T) and DF(V) cells, however, only in DF(T) cells was caspase 3 activity reduced when incubated with 17β-oestradiol and embelin. 17β-oestradiol regulated IAP and IAP antagonists mRNA expression differently between DF(T) and DF(V) cells under unwounded and mechanically wounded conditions. The majority of the IAPs increased in DF(T) cells with 17β-oestradiol treatment, however, in DF(V) cells, expression ether remained the same or decreased with the exception of XIAP in unwounded and DIABLO in wounded DF(V) cells, which increased with 17β-oestradiol. Suggesting that 17β-oestradiol is able to prevent apoptosis in DF(T) cells and reduce caspase 3 activity potentially through increased XIAP levels.

This thesis therefore concludes that DF(T) and DF(V) cells possess different characteristics which could explain differences in wound healing responses. XIAP is an important IAP in maintaining cell viability, with stronger effects in DF(T) cells compared to matching DF(V) cells. Whilst 17β-oestradiol protects DF(T) and DF(V) cells, other mechanisms are possibly in place in DF(V) cells which helped protect against embelin. 17β-oestradiol potentially reduced caspase 3 activity when in the presence of embelin via increased XIAP expression. Further studies are required to fully understand the roles of these individual IAPs and their antagonists in wound healing responses.

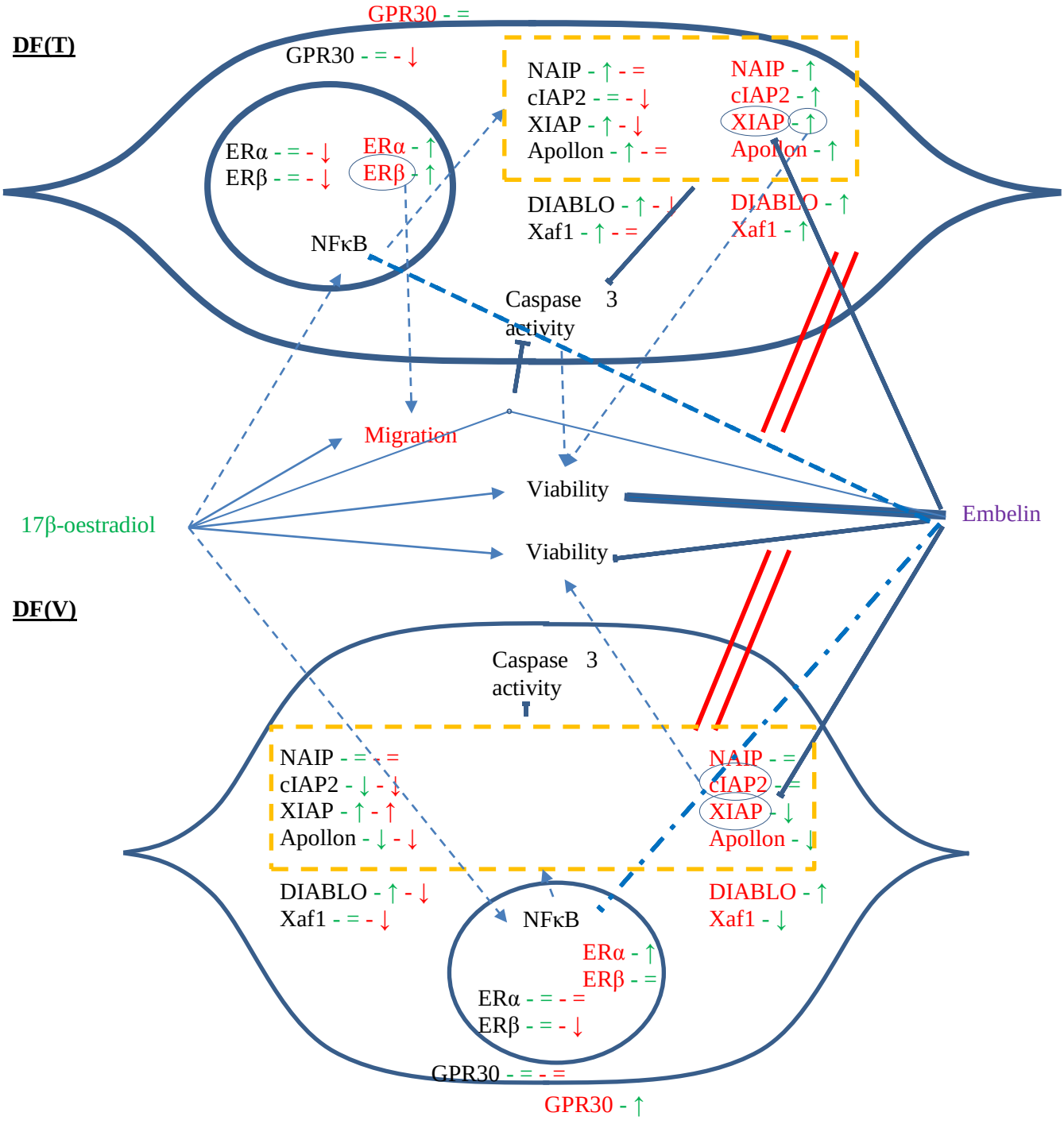


Figure 6.1: Summary diagram of the effects of 17β-oestradiol and embelin on DF(T) and DF(V) cells

A summary on the effects of wounding (red), 17β-oestradiol (green) and embelin (purple) on DF(T) and DF(V) cells in terms of mRNA expression of IAPs and their antagonists and oestrogen receptor expression concluded from this study (bold lines), and potential interactions (dashed lines).

6.3 Further Studies

From this study, it has been established that human dermal fibroblasts derived from similar regions of the skin from the same donor that contain either terminal or vellus hair follicles, exhibit different properties in culture. To further understand the differences between DF(T) and DF(V) cells in wound healing responses a comparison of αSMA expression in DF(T) and DF(V) cells should be conducted. αSMA is a marker for myofibroblasts which are important in contraction and relates to scarring. Immunofluorescence using antibodies against αSMA or qRT-PCR would establish whether there is a difference between DF(T) and DF(V) cells since scarring is more observant in non-hairy skin compared to hairy skin, therefore to study if there is a differences in contraction between the two cell types, would help establish potential mechanisms to reduce the effect of scarring. A collagen contraction assay could also be used to determine whether DF(T) or DF(V) cells differ in their contractile abilities. TGFβ1 secretion can also be analysed as this is an important growth factor in the production of collagen. A collagen detection kit could establish collagen synthesis in both DF(T) and DF(V) cells and if there is a difference between the two cell types. Using an ELISA assay, TGFβ1 secretion between DF(T) and DF(V) cells can be determined in response to mechanical wounding and 17β-oestradiol. qRT-PCR will also help establish TGFβ1 transcription between DF(T) and DF(V) cells. qRT-PCR would also establish transcription levels for adhesion molecules such as ICAMs. Establishing differences in collagen production and how it is regulated in DF(T) and DF(V) cells could provide potential therapeutic targets to control collagen secretion and reduce scarring.

17β-oestradiol also modulated DF(T) and DF(V) cells differently in terms of migration with no effect on proliferation. Therefore, to fully understand how and which aspects of wound healing is improved by 17β-oestradiol in both DF(T) and DF(V) cells, collagen synthesis, contractile properties and adhesion of these dermal fibroblasts could be assessed in the presence of 17β-oestradiol. By determining which oestrogen receptor is involved could provide

therapeutic targets in improving wound healing. This can be achieved by using specific ERα and ERβ agonists or silencing oestrogen receptor genes using siRNA. Whilst oestrogen treatment has positive impact on certain cells in the skin by increasing skin elasticity and in bones by protecting against erosion and activating osteocytes, and detrimental impact on others (such as breast cancer), topical application could help localise treatment to affected areas.

17β-oestradiol mainly increased IAP mRNA levels (XIAP, NAIP, Apollon and cIAP2) and their antagonists (DIABLO and Xaf1) in DF(T) cells and reduced them in DF(V) cells. The main differences between DF(T) and DF(V) cells were seen particularly in XIAP and Xaf1 mRNA levels in wounded DF(T) and DF(V) cells. This could be due to NFκB regulation, therefore using cell fracture, nuclear contents may be separated from the cytoplasm. NFκB activation can be assessed in DF(T) and DF(V) cells to look at possible links between NFκB regulation and IAP expression. Since IAPs are regulated through NFκB activation, differences between DF(T) and DF(V) cells would help establish whether that is the cause for differences in IAP levels which then can be targeted.

To determine which genes (in particular those responsible for wound healing) are regulated by 17β-oestradiol in DF(T) and DF(V) cells, and whether there is a difference between the two, ChIPSeq and gene microarray can be carried out on DF(T) and DF(V) cells treated with 17β-oestradiol. This would help establish which genes are important in a wound healing response with minimal consequences such as scarring, and therefore allow for gene targeting. This could be via epigenetics, where genes are targeted to become inactive.

Incubating DF(T) and DF(V) cells with embelin reduced viability, however the difference in reduction between DF(T) and DF(V) cells would suggest that DF(V) cells have other mechanisms in place that protect cells. Therefore, to determine whether the other IAPs are important in cell viability, DIABLO mimetics could be used to block IAP function. Theoretically, if the difference between DF(T) and DF(V) cell viability was due to other IAPs in DF(V) cells protecting the cell, cell viability should be reduced equally when incubated with

DIABLO mimetics, or if each individual IAPs is silenced using siRNA. By understanding which IAP is involved, it could potentially be targeted and upregulated when cell death needs to be controlled.

The fact that 17β-oestradiol blocked caspase 3 activity in DF(T) cells but not DF(V) cells when treated with embelin would suggest that there is another mechanism in place in DF(V) cells that didn't allow caspase 3 activity to be affected. This could potentially be due to XIAP expression and the effect of 17β-oestradiol on XIAP in DF(T) and DF(V) cells. To solidify this claim siRNA for XIAP may be used on DF(T) and DF(V) cells; since protein expression between DF(T) and DF(V) cells was the same, silencing the gene should cause no changes in XIAP expression when treated with 17β-oestradiol. Therefore the effect of embelin and 17β-oestradiol together should be the same between DF(T) and DF(V) cells.

The IAP Survivin has been associated with not only inhibiting apoptosis, but also in promoting cell proliferation. This IAP is capable of translocating into the nucleus which has been associated with its dual function. XIAP is another IAP that can also be found in the nucleus, therefore, it is possible that XIAP may have other roles such as promoting proliferation and migration. An MTT assay or a BrdU assay may be used to measure proliferation properties. The effect of silencing the XIAP gene on cell proliferation can be determined by a BrdU assay. This will help determine whether XIAP also possesses other functions.

Embelin has been shown to block NFκB activation, which is important in IAP regulation. Therefore it is important to determine whether embelin is not only directly blocking XIAP, but also regulating other IAPs. Using qRT-PCR on DF(T) and DF(V) cells treated with embelin will establish whether embelin does indeed regulate other IAP expression. Embelin was designed to bind to XIAP in a similar way in which DIABLO binds to XIAP. DIABLO can also be found in the nucleus, therefore if embelin affects NFκB, DIABLO might also be able to regulate NFκB. Silencing the DIABLO gene and measuring NFκB activity via Western blotting might help establish whether DIABLO has another

function aside from antagonising IAPs. The expression of DIABLO in DF(T) and DF(V) cells and skin from terminal and vellus hair bearing skin showed high expression of DIABLO. Suggesting again that DIABLO may have another role in human skin. More studies need to be done on the importance of DIABLO alone in skin biology. This could be done on transgenic mice that have the DIABLO gene knocked out. This will show whether DIABLO has any phenotypical effects on the skin. In dermal fibroblast cells, silencing DIABLO by siRNA would establish whether this is sufficient enough to reduce cell viability. If cell viability is not affected in DF(T) and DF(V) cells, it would suggest that DIABLO has other roles.

A wound healing response is not confined to one cell, in the skin, keratinocytes and endothelial cells are important in wound healing. Therefore it is important to understand the interactions that occurs between different cells, and how these cells affect or potentially are affected by DF(T) and DF(V) cells in the presence and absence of 17β-oestradiol. Migration effects can be observed after keratinocyte or endothelial cell cultures are treated with conditioned medium from DF(T) and DF(V) cells at different time points, up to 48 hours, and qRT-PCR carried out on the keratinocytes and/or endothelial cells. Co-cultures between the different cell types would establish if DF(T) and DF(V) cells have different effects on keratinocytes and endothelial cells in terms of migration and proliferation.

It is important to continue these studies to help establish the mechanisms that are involved in DF(T) and DF(V) cells, which could therefore help identify therapeutic targets that would improve wound healing responses. Understanding the mechanisms and being able to target specific pathways which contribute to improved wound healing would be able to be applied to patients with chronic non-healing wounds and to help improve scarring effects, especially in postmenopausal women.

Chapter 7

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8 Appendix

8.1 Solution Protocol

8.1.1 Celestine blue

A 5% aqueous solution of ammonium iron sulphate (500ml) was added to 2.5g of Celestine blue. After boiling for 3 minutes, the solution was allowed to cool, and filtered (grade 595 filter paper) before the addition of 70ml glycerol.

8.1.2 Picric acid/ ethanol

A saturated alcoholic solution of picric acid (5ml) was mixed with 300 ml absolute ethanol.

8.1.3 Picro-indigo carmine

A saturated aqueous solution of picric acid (300ml) was mixed thoroughly with 1g of indigo-carmine; this solution was filtered before use.

8.1.4 Safranin

6g of Safranin was dissolved in 300ml of a 1:1 ethanol:distilled water solution. The solution was filtered before use.

8.1.5 Scott's tap water

2g of sodium hydrogen carbonate and 20g of magnesium sulphate were dissolved in 1L distilled water, filtered and stored at 4˚C until used.

8.1.6 Gill's haematoxylin

Dissolve 16.7ml of ethylene glycerol in 50ml of distilled water. Add 0.13g of haematoxylin, 0.013g of sodium iodate, 1.33g of aluminium sulphate and 1.33g of glacial acetic acid. Stir for 1 hour at room temperature, filter before use.

8.2 Abstracts

8.2.1 7th World Hair Congress

Differences in the upregulation of inhibitor of apoptosis proteins (IAPs) in human dermal fibroblasts derived from hair-bearing and non hair-bearing skin in a scratch wound assay

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Observational studies describe rapid donor site healing when scalp skin is used for split-thickness skin grafting, suggesting that wounds heal better from hair-bearing skin. This has mainly been attributed to the hair follicle, but studies also suggest that dermal fibroblasts from different regions exhibit phenotypic differences. Recently the role of IAPs, a family of antiapoptotic proteins that inhibit the activity of caspase proteins, in wound repair has been highlighted.

Using matched patient skin samples (n=3), we cultured dermal fibroblasts from hair-bearing scalp skin and adjacent skin that does not contain terminal follicles. In a comparison of fibroblasts derived from the same patients, at the same passage number, those derived from hair-bearing skin grew at a much slower rate over a 12 day period, compared to their adjacent counterparts.

Using a scratch wound assay we compared the expression of a panel of IAPs (NAIP, c-IAP2, XIAP and Apollon) and their antagonists (XAF1 and SMAC/DIABLO) immediately after scratching and 24 hours later using immunocytochemistry quantitated by ImageJ.

Fibroblasts from hair-bearing skin demonstrated an increase in the expression of NAIP, Apollon and SMAC/DIABLO immediately after scratching; all returned to control levels after 24h. In contrast, only an upregulation in the expression of SMAC/DIABLO was observed in matching fibroblasts from non hair-bearing skin.

These results provide further evidence for important phenotypic differences in human dermal fibroblasts from skin containing either terminal or vellus hair follicles. An immediate upregulation of the expression of IAPs in fibroblasts from hair-bearing skin suggests that they have better mechanisms in place for cell survival following wounding, which may contribute to the superior wound healing observed in hairy skin. Further studies are required to determine if fibroblasts from hair bearing skin significantly contribute to the wound healing response, which in turn may lead to improved therapies for chronic non-healing wounds.

8.2.2 Skin and Bone Conference

Human Dermal Fibroblasts Cultured from Different Anatomical Hair Bearing Skin Regions Respond Differently to Wound Healing Stimuli by Altered Expression of Oestrogen Receptors and the X-linked Inhibitor of Apoptosis Protein

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Human wounds heal better in skin containing large, pigmented terminal hair follicles e.g. scalp. Although this may be attributed to hair follicle stem cells, dermal fibroblasts from different anatomical regions exhibit phenotypic differences. Therefore, we compared matching fibroblasts derived from terminal and vellus (small, un-pigmented follicles) hair bearing skin of the same donor. Tissue repair requires tight control of cell proliferation, migration and apoptosis; animal studies have shown inactivation of the X-linked inhibitor of apoptosis protein (XIAP) impairs wound healing and processing of XIAP after injury is gender-specific and may be influenced by endogenous oestrogen. However, the relationship between oestrogen and XIAP in human skin has not been studied.

Dermal fibroblasts (DF) were cultured from female pre-auricular skin containing (T) terminal (scalp) or (V) vellus (facial) hair follicles. Cell morphology was analysed using FACS, and cell viability compared in the presence of 17β-oestradiol and the XIAP inhibitor embelin. Migration following mechanical wounding of cell monolayers was compared in the presence of 17β-oestradiol (1-100nM) and changes in mRNA expression of oestrogen receptors (GPR30, ERα and ERβ) and XIAP quantitated by qRT-PCR.

DF(V)s were smaller and more granular than corresponding DF(T)s. The XIAP inhibitor reduced cell viability in both, while 17β-oestradiol counteracted this effect. Only DF(T)s increased cell migration in response to 17β-oestradiol. The expression of all oestrogen receptor mRNA decreased in DF(T)s following mechanical wounding; only ERβ expression decreased in DF(V)s. ER expression was not modulated by 17β-oestradiol in intact fibroblasts, but 17β-oestradiol increased ERα and ERβ in wounded DF(T)s and increased GPR30 and ERα in wounded DF(V)s. The expression of XIAP mRNA was increased in intact cultures of both cell types in the presence of 17β-oestradiol; however, following wounding while 17β-oestradiol increased XIAP expression in DF(T)s, 17β-oestradiol decreased XIAP expression in DF(V)s.

These results demonstrate significant differences between dermal fibroblasts from different anatomical regions of the same individuals. Accelerated migration and an increase in XIAP expression via ERβ in DF(T)s would support previous animal studies showing oestrogen promotes cutaneous wound healing via ERβ. Further elucidation of the different signalling pathways may lead to improved therapies for chronic non-healing wounds.